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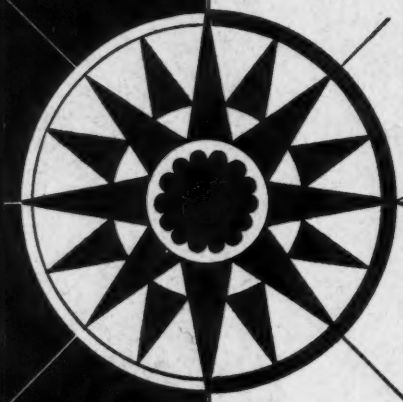

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ENDOCRINOLOGY

VOLUME 39 AUGUST, 1946 NUMBER 2

INHIBITORS OF HYALURONIDASE IN BLOOD SERA AND THEIR EFFECT ON FOLLICLE CELL DISPERSAL[1]

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SINCE HYALURONIDASE, an enzyme prepared from mammalian testes or spermatozoa, was found capable of readily dispersing the follicle cells of freshly ovulated mammalian ova without apparent injury to the egg (McClean and Rowlands, 1942; Fekete and Duran-Reynals, 1943; Leonard and Kurzrok, 1945), new concepts on problems of fertility and sterility have arisen. These observations have led to the suggestion that the relatively large numbers of sperm in an ejaculate are necessary to attain a sufficiently high concentration of hyaluronidase in order that the tightly adhering follicle cells can be removed for sperm penetration and in time before degeneration of the ovum. Rowlands (1944) succeeded in artificially inseminating and fertilizing rabbits with dilute sperm suspensions provided the enzyme from killed sperm was added. Without the enzyme, no fertilization occurred. Leonard and Kurzrok (1945) reported that it was possible to artificially inseminate and fertilize women by adding bull-testis hyaluronidase to the semen in cases which did not respond to semen alone. Joel and Eichenberger (1945) and Werthessen et al. (1945) demonstrated that in ejaculates of men, the amount of hyaluronidase in the semen was proportional to the concentration of spermatozoa.

The problem of sterility has been investigated also from the viewpoint of anti-hyaluronidase activity (Leonard andKurzrok, 1945). Not only was it possible to immunize rats against bull-testis hyaluronidase and show that the immune serum would prevent denuding of the eggs by the enzyme *in vitro*, but also it was observed that normal rat serum contained appreciable amounts of an inhibiting substance.

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This report will show that blood sera of different animals have varying amounts of hyaluronidase inhibitors, and that hyaluronidase from a different source reacts differently to these inhibitors.

METHODS

One preparation of hyaluronidase made from bull testes by Dr. Erwin Schwenk[2] and one prepared from rat testes were used. The latter was made by grinding the testes with sand and extracting with Ringer's solution acidified to approximately pH 5 with acetic acid. After standing in the ice box over night, the extract was centrifuged, the supernatant fluid neutralized to approximately pH 7 with .1N NaOH in Ringer's solution, and dilutions made so that 1 cc. = 1 gm. of fresh tissue. Toluene was added as a preservative.

Eggs were secured from young adult female rats. In the early evening, vaginal smears were made, and those rats showing predominantly nucleated epithelial cells were marked for the recovery of the eggs on the following morning. Such females usually exhibit extreme lordosis on gently pressing the pipette against the cervix. Placing the females with vasectomized bucks was not necessary although eggs were recovered on every occasion that a vaginal plug was formed and in about 75% of the time when no plugs were found or when the females were not placed with the males. The ova were removed from the oviducts with the aid of a binocular dissecting magnifier following a procedure previously described (Rowlands 1942).

Serum was obtained from the rat, rabbit, fowl, cow, horse, human, and whole blood from *Limulus polyphemus*, (the king crab).

The following method for assaying hyaluronidase activity by the rat-ova test (dispersal of the follicle cells) was adopted and it has given repeatedly satisfactory results. The eggs removed from each oviduct were placed in the separate concavities of a duplex depression slide, washed in Ringer's solution, and covered with .1 cc. of this solution. The slide was kept in a petri dish together with a piece of moistened filter paper when not under examination. At least 3 eggs were placed in each chamber. If fewer eggs were obtained from any one oviduct they were redistributed. The eggs usually adhere in a stringy mass but by the use of iridectomy scissors a given number can be cut out and transferred with a small glass pipette without fear of removing an appreciable number of follicle cells.

A series of enzyme dilutions in Ringer's was prepared and .1 cc. added to each group of eggs so that the total volume of fluid was .2 cc. The enzyme was stirred in with a needle and if the eggs were suspended in the surface film, they were gently pushed to the bottom of the depression slide. The slides were examined at intervals of $\frac{1}{2}$, $\frac{3}{4}$, 1, $1\frac{1}{2}$, and 2 hours and longer if necessary. The end point was established when 2 of 3 eggs, or 50% of a greater number (not exceeding 8), had lost their follicle cells to a degree varying from complete denudation to where not more than a single layer of follicle cells remained. Previous experience on the variation of degree of follicle cell dispersal has led us to adopt this end point and it has proven quite satisfactory. *A unit of hyaluronidase by the rat-ova test was defined as the amount of hyalu-*

[2] The bull-testis hyaluronidase preparation was provided through the courtesy of Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, New Jersey.

ronidase in .2 cc. of Ringer's solution which gave an end point, as defined above, within ¾–1 hour. In the experiments to be reported, this unit of hyaluronidase was found convenient because it allowed sufficient time to run several determinations in a day and to read the results with some degree of accuracy. The greatest error seemed to occur in the repeated weighings of small quantities of the enzyme. All experiments were conducted at room temperature.

In testing the inhibitory action of the sera, .1 cc. was first added to the ova, after removing the Ringer's solution, and a unit of hyaluronidase, dissolved in .1 cc. of Ringer's, was stirred in. Control tests were made simultaneously, using either Ringer's or rat serum diluted 1:10. The repeated use of controls was made to circumvent gross errors in weighing the enzyme on each day the tests were run. In some experiments, the serum to be tested was diluted 1:10 because previous results had shown that rat serum completely inhibited a "unit" dose of hyaluronidase. Comparisons of the time required to reach the end point which was similar to that used in the assay of the hyaluronidase, were indicative of the relative inhibitory power of the sera.

RESULTS

The unit dose of the bull-testis hyaluronidase preparation was .033 mg. as determined by the method outlined above. In 27 weighings of 1 mg. quantities of hyaluronidase for preparation of the unit doses during the course of the following experiments, only 6 failed to disperse the follicle cells within the specified time.[3]

The results in Table I indicated that the sera of the several species tested, did not inhibit the bull-testis hyaluronidase to the same extent. Roughly, the sera could be arranged in a decreasing order of inhibitory power: (1) rat, chicken, (2) rabbit, horse, (3) *Limulus*, human (4) cow. It was not particularly advantageous to make comparisons of the diluted test sera with diluted rat serum as the control but when these experiments were begun this was not known. By diluting the test sera, it was found that in the case of chicken serum, the enzyme action could be demonstrated. Dilution of the cow serum had barely an appreciable effect in decreasing the already weak inhibitory action. Although undiluted rat serum completely inhibited the unit dose of hyaluronidase, it was found that larger doses of the enzyme could disperse the follicle cells in the presence of rat serum. .20 mg. of the bull-testis hyaluronidase dispersed the cells in 22 hours (2 cases) whereas 1 mg. dispersed the cells in ½, 1 and 1 hour respectively in 3 cases.

When rat-testis hyaluronidase was used, very little difference in the inhibitory property of the sera was noted. .1 cc. of rat-testis extract was the quantity used. Table II shows that the sera only

[3] Standardization of 2 different samples of hyaluronidase, prepared by Dr. E. Schwenk, by 2 different assay methods also indicated the usefulness of the rat-ova test for biological standardization. Samples A and B assayed 4.1 and 16.0 turbidity-reducing units per mg. respectively by testing the enzyme's ability to prevent the formation of a precipitate when acidified horse serum and hyaluronic acid were combined (E. Schwenk). By the rat-ova test, .033 mg. of sample A and .0083 mg. of sample B denuded the eggs in the required time (¾–1 hour). Thus B was 4 times more potent than A by 2 different asasy methods.

TABLE I. INHIBITORY ACTION OF SERA FROM DIFFERENT SPECIES ON THE EFFECT OF BULL-TESTIS HYALURONIDASE

Control	Dispersal time (hours)	Experimental	Dispersal time (hours)
		Rat Serum	
Ringer's	¾, 1, 1[1]	no dil.	0, 0, 0
Rat Serum		*Chick Serum*	
dil. 1:10	3, 5½, 5½	dil. 1:10	3, 7, 5½
Ringer's	1, 1½	dil. 1:10	7, 5½
Ringer's	1, 1	no dil.	0, 0
Rat Serum		*Rabbit Serum*	
dil. 1:10	4, 4, 2, 2	dil. 1:10	1, 1, 1, 1
Ringer's	½, ¾, 1	no dil.	4½, 6½, 8½
Rat Serum		*Horse Serum*	
dil. 1:10	2½, 2, 5½	dil. 1:10	1, ¾, ¾
Ringer's	1, ¾	dil. 1:10	1, 1
Ringer's	½, 1	no dil.	7, 4
		Human Serum	
Ringer's	¾, ¾, 1½, 1, 1	dil. 1:10	1, 1, 2½, 1, 1½
Ringer's	1, ¾	no dil.	3½, 4
		Limulus Blood	
Ringer's	½, 1	no dil.	4½, 4
Rat Serum		*Cow Serum*	
dil. 1:10	3, 1½, 1½	dil. 1:10	2, ¾, ¾
Ringer's	1, 1	dil. 1:10	1, 1½
Ringer's	¾, 1½	no dil.	1½, 2½

[1] The dispersal time for the control and experimental tests in each case is noted in corresponding positions in each line.

TABLE II. INHIBITORY ACTION OF SERA FROM DIFFERENT SPECIES ON THE EFFECT OF RAT-TESTIS HYALURONIDASE

Control	Dispersal time (hours)	Experimental	Dispersal time (hours)
		Rat Serum	
Ringer's	¾, 1[1]	dil. 1:10	1½, 1½
	1, 1	no dil.	2½, 2
		Chick Serum	
Ringer's	½, ½	no dil.	¾, 1
		Horse Serum	
Ringer's	¾	dil. 1:10	1½
	½, ¾	no dil.	½, ¾
		Human Serum	
Ringer's	½, ½	no dil.	1, 1
		Cow Serum	
Ringer's	¾, ½	dil. 1:10	2, 1
	1, 1	no dil.	2, 2

[1] The dispersal time for the control and experimental tests in each case is noted in corresponding positions in each line.

slightly inhibited this enzyme preparation. The best inhibitory action succeeded in only doubling the time necessary for denuding the ova when compared with the effect of the enzyme in Ringer's solution.

Previously, it had been shown that serum from rats immunized against the bull-testis enzyme inhibited the enzyme very markedly. This observation was confirmed. However, if rat-testis hyaluronidase was employed to disperse the follicle cells in the presence of anti serum against bull-testis hyaluronidase, the time required was identical to that obtained when normal rat serum was used. Dispersal of the follicle cells was obtained in ¾ and 1 hour with immune serum, and ¾ and 1 hour with the control rat serum. The immunity to the enzyme was thus observed to be specific for the source of the enzyme used as the antigen.

DISCUSSION

The discovery of the variation in the inhibitory power of normal blood sera on hyaluronidase and the different results obtained with hyaluronidase of different origin has laid a basis for investigation on the possible use of blood sera to prevent conception. An attempt was made to prevent conception in 7 female rats by introducing approximately .2 cc. of rat serum into each uterine horn (via the vagina) when the rats were in heat. They were placed with males immediately afterwards. Copulations occurred within 8 hours and all the females conceived. The results obtained by the *in vitro* experiments indicated that rat-testis hyaluronidase was inhibited only to a slight degree by rat serum and presumably not sufficiently to prevent conception in the normal animal.

The fact that blood serum of mammals will inhibit the enzyme hyaluronidase has been reported but entirely different types of test methods were used. The addition of rabbit serum to tubes containing strains of virulent encapsulated streptococci and hyaluronidases prevented decapsulation of the organisms by the enzyme (McClean, 1942). Sera of several species of mammals were found not to be equivalent in this inhibitory power but details of the experiments were not given.

By testing the effect of rat-testis extracts, containing hyaluronidase, on the enhanced spreading of vaccine virus through the skin of rabbits, Duran-Reynals (1932) illustrated that normal rabbit serum has an inhibitory effect although he did not mention this in the text of his paper. Hobby et al. (1941) reported that human and rabbit serum were able to inhibit bacterial hyaluronidases from hydrolyzing hyaluronic acid, and forming the usual reducing substances. On the other hand, McClean and Hale (1941) obtained no inhibition of the formation of the reducing substance (N-acetyl glucosamine) when he used other bacterial hyaluronidases in the presence of rabbit serum.

McClean (1942) believes that this inhibitor is associated with the pseudo-globulin fraction of blood serum. This has not been tested by

the method involving dispersal of the follicle cells. It has been noted, however, that egg albumen and heparin would inhibit hyaluronidase by the rat-ova test to approximately the same extent as human serum. The nature of blood inhibitors has yet to be conclusively identified.

Attempts to find increased amounts of an inhibitory substance in blood sera of 9 women known to be sterile were made. A clinical diagnosis for sterility was made in each patient on other grounds by one of us (R.K.). In no case was there any noticeable lengthening of the time of dispersal of the follicle cells indicating an increased amount of an inhibitor. The dispersal time varied from 3 to 5 hours, with an average of 4 hours. If some types of sterility in a woman could be due to an increased amount of a normal or an induced inhibitor (antibody to human-sperm hyaluronidase) it would appear, in the light of our experiments, that studies must be made using human-testis hyaluronidase to disperse the follicle cells.

SUMMARY

Bull-testis hyaluronidase acting to disperse the follicle cells of rat ova *in vitro* was inhibited by blood sera of the following animals, roughly in a decreasing order of effectiveness: (1) rat, chicken (2) rabbit, horse (3) *Limulus*, human (4) cow. When rat-testis hyaluronidase was used, only a small uniform inhibitory action by these sera was observed. The failure of rat serum to prevent conception when introduced into the rat uterus was explained by these results.

The inhibitory activity of rat serum on bull-testis hyaluronidase can be overcome by increasing the amount of the enzyme. Rat serum containing antibodies against bull-testis enzyme did not inhibit rat-testis enzyme to a degree greater than that obtained with normal rat serum.

Women, diagnosed as sterile, had normal amounts of inhibitor in their blood serum.

A method for standardization of hyaluronidase by the rat-ova test is presented. A *rat-ova unit of hyaluronidase* is defined as the amount of enzyme in .2 cc. of Ringer's solution which will disperse the follicle cells of 2 of 3 or 50% of a greater number of ova (not exceeding 8) within ¾–1 hour. The follicle cells are considered dispersed when not more than a single layer of cells remain surrounding an ovum.

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EFFECT OF PURIFIED GONADOTROPES ON THE ANDROGEN-SECRETING ABILITY OF TESTES OF HYPOPHYSECTOMIZED COCKS[1]

A. V. NALBANDOV, ROLAND K. MEYER AND W. H. McSHAN
From the University of Illinois and the University of Wisconsin

IN AN EARLIER study Nalbandov and Card (1943) noted that Leydig cells did not disappear from the testes of cocks following hypophysectomy. In spite of this, the comb and the wattles of hypophysectomized males regressed completely and became capon-like in appearance. This observation led to the question of the endocrine function of the intertubular elements in hypophysectomized males. The observation that certain preparations of FSH caused comb growth, while certain LH preparations did not, made it desirable to reexamine the question of the origin of androgens in avian testes.

REVIEW OF LITERATURE

Most available data seem to indicate that the Leydig cells are the only source of male sex hormone. There are some data which make it appear possible that the seminiferous tubules are at least in part responsible for the secretion of androgen.

As evidence of the participation of the seminiferous tubules in the formation of androgen the following work may be cited.

Nelson (1937) found that rat testes which were confined experimentally to the abdomen secreted much less androgen than did scrotal testes. This was especially true of rats which had been cryptorchid for prolonged periods of time (240 days or longer). Nelson explained these results on the assumption that cryptorchism had caused a reduction in the gonadotrophic hormone secretion by the pituitary. This was borne out by the finding that the injection of gonadotrophic hormone led to an increase in the androgen secretion by cryptorchid testes. Also, the pituitary gland of the cryptorchid animals showed castration changes.

Moore (1944), repeating parts of Nelson's work, found that in rats, experimental cryptorchism between the ages of 20 and 80 days re-

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[1] This study was planned and executed as a cooperative project between the two institutions involved. Support of the University of Illinois Research Board and the Wisconsin Alumni Research Foundation is gratefully acknowledged. Dr. J. B. Schooley, formerly of Difco Company, Dr. C. F. Church, of the Squibb Institute, and Dr. W. F. Wenner of the Upjohn Company kindly made some of the preparations available to us for use in this study.

duced the amount of male sex hormone significantly (as measured by the seminal vesicle test). The magnitude of response of scrotal testes to injected gonadotrophins was also greater than that of abdominal testes. Unlike Nelson, Moore failed to find any impairment of the androgen-secreting ability of the testes of chronologically older rats, or of cryptorchids of long standing. These findings leave the possibility open that the seminiferous tubules may have contributed some hormone with androgenic properties.

Hanes and Hooker (1937) using the capon comb test, assayed the scrotal and the abdominal testes of boars and found that much less male sex hormone was present in abdominal than in scrotal testes. They gave no information about the age of the donors nor about the state of their accessories.

Kudriashov (1931) correlated the degree of testicular destruction caused by vitamin E deficiency with the hormone output of the testes, as measured by the maintenance of the accessories. He found that the degeneration of the accessories (seminal vesicles first, prostate next), began as soon as the spermatogenic activity of the testes was impaired. In the last stages of vitamin E deficiency, when the seminiferous tubules had degenerated completely, the accessories showed castration changes, but the Leydig cells were still numerous and appeared to be in a secretory state. With continued vitamin E deficiency even the Leydig cells degenerated, but by that time the accessories were so completely atrophic that they could not show any further effect of lack of androgen.

Breneman (1935 & 1936) found that in the chick, only the injection of FSH leads to comb growth, while LH had no such effect. This suggested that in the chick the tubules are the site of male hormone formation rather than the interstitial tissue. Seemingly supporting evidence will be presented in the body of this paper.

Pfeiffer and Kirschbaum (1943) found that in the male sparrow there was no correlation between the deposition of melanin in the bill (which can be brought about in the castrated male by the injection of androgen), and the presence of Leydig cells in the intertubular tissue. In fact, these authors could find no Leydig cells at all in normal males in breeding state. This leads to the possibility that in this case the androgen originated in the germinal epithelium. However, when such males were injected with PMS, Leydig cells in large numbers appeared in the intertubular tissue. With lower doses of PMS the germinal epithelium was preferentially stimulated with very little Leydig cell development, but here too the level of androgen secretion was high. In young chicks Leydig cells were readily formed when PMS was injected, but the amount of androgen secreted was not increased appreciably.

Of the evidence in favor of the Leydig cells as the only source of androgen, only the most recent is cited here (for a more complete discussion see Burrows 1945).

Witschi, Levine and Hill (1932) and Johnston (1934) destroyed the seminiferous epithelium of rats with X-rays or radium, leaving the more resistant interstitial tissue undamaged. This treatment did not cause the atrophy of the accessory glands suggesting that the destruction of the seminal epithelium did not lead to a reduction in androgen secretion.

In a study of testes from sexually immature calves up to and including the testes of mature bulls, Hooker (1944) found no correlation between the androgen content of the testes and the state of development of the seminiferous tubules. There was a strong direct relationship between the changes in androgen content of the testes and the number, but especially the vacuolation of the Leydig cells.

Pollock (1942) showed that histochemical reactions indicated the presence of steroid substances in the interstitial cells but not in the seminiferous tubules.

The work presented in this paper adds some new information to the question of the source of androgen in the testes. The results to be discussed are considered especially significant in view of the peculiar suitability of the hypophysectomized cock for the assay of hormones stimulating the secretion of androgen.

PLAN OF EXPERIMENT

The animals used in this study were White Leghorn male chicks which were hypophysectomized when they weighed between 500 and 600 grams and were between 60 and 70 days old. During a post-operative recovery period of 14 days the head region assumed the pallor characteristic of the hypophysectomized cock. The combs of all totally hypophysectomized birds regressed completely and became capon-like (dry and scaly) in appearance. Birds showing continued comb growth or even slight indications of coloration about the face or head region were discarded as being incompletely hypophysectomized.

The remaining birds were injected with FSH, LH, and other gonadotrophic preparations from various sources. The assumption was made, that if the prevalent theory concerning the effect of FSH and LH upon the male gonad is the same for the bird as it is for the mammal, FSH should stimulate growth of the germinal epithelium, while LH should cause the growth of the intertubular tissue, especially the Leydig cells. With the head furnishings of the cock, serving as an accurate indicator of any male hormone which may be produced as a result of these treatments, it is an easy matter to determine the extent to which these two hormones are capable of stimulating production of androgen. It would also be possible to determine the role of the two components of the testes with regard to the production of male sex hormone.

HORMONE PREPARATIONS AND DOSAGES USED

The study was begun with FSH and LH (Luteogen) obtained from Difco and Company. These two hormones were injected into hypophysectomized males in dosages of "1 hen unit" (see Nalbandov and Card, 1946, for details). Some of the Luteogen preparations used (S-95 and S-96) were free from

prolactin, while RX 43255 and RX 361606 were very rich in prolactin. This information was not available at the time when the experiments were conducted.

The Wisconsin preparations were received at Illinois accompanied by assays done on intact immature female rats. It was felt that the rat response gave little, if any, indication of the possible potency of these hormones in the hypophysectomized cock. For this reason it was decided to inject the Wisconsin preparation in dosages well beyond the possible minimum effective dose of these hormones. Thus we did not need to be concerned with the possibility of negative results which subsequently might have to be ascribed to insufficient dosages. For these reasons the Wisconsin FSH and LH preparations were injected in dosages of 5 grams equivalent of the acetone-dried powder.

The Squibb hormones, thylakentrin (FSH) and metakentrin (LH), prepared by the method of Greep, Van Dyke and Chow (1942), were not available in sufficiently large quantities to be used on a large number of animals. Since no bio-assays were available on either of these hormones, the dosages to be injected were decided arbitrarily on the basis of the total amount of hormone available. Metakentrin was injected at the rate of 32 mgs. per male, while the thylakentrin was given at the rate of 50 mgs. per male during the 8-day experimental period.

The gonadogen (Upjohn) and the chorionic gonadotrophin (Upjohn) were the standard commercial grades and were injected at the rate of 1000 I.U. and 1250 I.U. per male respectively.

The experimental period in all cases lasted eight days. The hormones were dissolved in water and 0.5 ml. of the solution was injected sub-cutaneously once daily. On the first, fifth, and ninth days of the experiment, the length and the height of the combs of the males were measured; the product of these two figures gave a comparative basis for determining the changes in the area of the comb. Autopsy was performed on the ninth day of the experiment. The testes were preserved in FAA, later weighed, cut at 8 mmu. and stained with Mallory's triple stain. The measurements of the components of the testes given in the tables were obtained in the manner described by Nalbandov (1945).

RESULTS

Difco FSH and LH (Luteogen). The initial experiments in which Difco hormones were used were not carried out at the same time as those involving hormones from other sources. Since all available supplies of the Difco preparations were used up in the initial tests, they could not be compared simultaneoulsy with the other hormone preparations. This circumstance does not invalidate the information obtained in these initial trials nor the conclusions to be drawn.

The androgen-producing ability of the Difco FSH and LH was checked on a total of eight males; only two males on each treatment are included in the final summary (table 1), because the other tests were conducted at different times and the cocks used differed from the rest in age and in the amount of hormone they received. However the results obtained with these males agree completely with those included in the tabulation.

The Difco hormones affected the testes of hypophysectomized cocks in a manner opposite to expectation (table 1). FSH caused a doubling of comb area, indicating that it was capable of causing the testes to secrete androgen. FSH also caused an increase in testes weight. LH, on the other hand, had no effect on either comb growth or testes weight.

These results tend to support the evidence presented by Breneman (1935) that in the chicken the tubules, which are under FSH control, secrete androgen, while LH did not appear to be capable of such action. This also agrees with the evidence to be presented later in this

TABLE 1. EFFECT OF DIFCO FSH AND LH PREPARATIONS UPON THE TESTES OF HYPOPHYSECTOMIZED COCKS

Treatment	Number of birds	Comb area		Testes weight (grams)	Tubule area[1]	Intertubular area[1]	Tubule diameter[1]
		Initial	Final				
RX 43732 (FSH)	2	16.3	32.0	0.775	2.00	0.58	25.08
RX 43255 (LH)	2	14.4	14.4	0.076	1.69	0.80	11.50
Uninjected controls	4	16.1	15.6	0.063	1.69	0.75	12.53
Normal controls	2	92.8	99.8	12.950	2.55	0.02	35.99

[1] A relative measure.

paper that PU does not cause secretion of androgen in spite of massive doses.

The paucity of data obtained with Difco material, and the inability to procure additional supplies from that source caused us to continue the work using FSH (1940, 1946) and LH (1943) preparations made by the method of McShan and Meyer. The hormones were prepared and assayed at the University of Wisconsin, while the work with hypophysectomized cocks was carried out at the University of Illinois.

Wisconsin Preparations. Four different FSH and three different LH preparations from that source were used in these studies. In addition, an unfractionated preparation, F 116A, which contained both FSH and LH, but relatively little of the latter, was used.

Only one FSH preparation (FSH 324) was available in 1945 when the first experiments were done, and it alone was tested simultaneously with the LH preparations and the other gonadotrophins. Later, other Wisconsin FSH preparations became available and results obtained with FSH 324 were checked with them. Three additional FSH preparations were tested early in 1946 in a manner duplicating the 1945 plan as closely as possible. To provide a check for these FSH preparations, two hypophysectomized males were injected with gonadogen and two with LH P325. Because of an outbreak of chicken pox, the number of available males was greatly reduced and only three males could be used on each of the three additional FSH preparations. Chronologically then, FSH 325 and Extract F 116A were

tested simultaneously with all the Wisconsin LH preparations, the metakentrin, thylakentrin, gonadogen, and the PU. FSH preparations 124, 534, and 5256 were tested later and separately. The available rat assay data for these and the other hormones is given in abridged form in table 2.

F 116A, which contained both FSH and LH, caused both comb growth and a big increase in testes weight (table 3).

FSH preparations 324, 534, and 5256 caused no comb growth, the comb remaining dry and the head region pale. All three of these hormones caused an increase in testes weight in cocks (table 3), and produced only follicles when injected into immature female rats (table 2).

TABLE 2. EFFECTS OF THE DIFFERENT GONADOTROPES ON IMMATURE FEMALE RATS

Hormone identification	Dose	Number of rats	Average ovarian weights (mgs.)	Ovarian response
FSH 534	1– 1.5 gms.[1]	3 & 6	34–60	all folls.
FSH 124	0.5– 1.0 gms.[1]	3 & 6	38–64	0.5 gms.—folls. 1.0 gms.—folls. and corpora 1.
FSH 5256	0.5– 1.0 gms.[1]	3 & 3	17–71	all folls.
FSH 324	0.5– 1.0 gms.[1]	6 & 6	34–85	all folls.
FSH RX43732	1.0– 5.0 mgs.	2 & 1	81–130	follicles & corpora 1
Thylakentrin	1.0–10.0 gms.	2 & 1	13–20	slight stimulation
F 116A	100 mgs.[1]	5	89	follicles & corpoa
LH P116BC	0.5 gms.[1]	2	13	slight stim.
LH P325	0.5 gms.[1]	2	11	slight stim.
LH R28A	0.5 gms.[1]	2	15	slight stim.
LH RX43255	1.0– 5.0 mgs.	2	11–17	all small follicles

[1] The amount injected was equivalent to the indicated amount of acetone dried pituitary powder. All other quantities are the actual amounts injected.

FSH 124 was quite toxic, causing apparent distress to two of three hypophysectomized cocks into which it was injected. One of the three males died twenty-four hours before his mates were autopsied. In this group FSH 124 caused an increase in comb size, and the faces of all three males were "flushed" on the second day after the first injection. The increase in testicular weight was less in this group than in the other males, probably partly because of the toxicity of the preparation. In rats, FSH 124 produced no corpora when given at the level of 0.5 grams equivalent, but at the level of 1.0 gram equivalent, two of the twelve pairs of ovaries contained "several corpora" (table 2).

On the basis of these results the conclusion is reached that FSH preparations 324, 534, and 5256 were completely free from LH since they induced neither comb growth nor a flushing of the faces and head regions of the injected birds. Nevertheless, they are biologically active since they increased testes weight considerably. FSH 124 was not free from LH since it induced first coloration of the head region and also comb growth in addition to an increase in testes weight.

Three Wisconsin preparations of LH were used in addition to the FSH hormones discussed above. LH preparations P 116BC, P 325, and R 28A all caused comb growth and an increase in testes weight (table 3).

On the basis of the measurements made to determine the effect of the various gonadotropes upon the testes components, it can be stated that the FSH preparations produced a significant increase in tubule diameter while LH has a lesser effect in this respect. FSH also in-

TABLE 3. SUMMARY OF EFFECTS OF VARIOUS GONADOTROPHIC PREPARATIONS ON THE TESTES OF MALE HYPOPHYSECTOMIZED CHICKENS

Hormones injected	No. of animals	Comb area Initial	Comb area Final	Testes weight (grams)	Tubule area[1]	Inter-tubular area[1]	Tubule di-ameter[1]
LH P 116BC	4	9.30	18.70	0.272	1.41	1.12	14.83
P 325	4	10.50	15.50	0.184	1.39	1.13	13.63
R 28A	4	10.90	21.30	0.232	1.35	1.15	15.30
FSH 324	4	9.61	8.90	0.300	2.05	0.76	15.94
5256	3	5.32	5.81	0.302	Measurements not obtained comb dry and scaly		
534	3	7.15	7.33	0.325	Measurements not obtained comb dry and scaly		
124	3	6.60	7.69	0.255	Faces "flushed." Combs red		
F 116A (a gonadotrope)	4	9.60	13.70	0.607	1.92	0.60	21.55
Thylakentrin (Squibb FSH)	4	11.30	17.60	0.961	2.03	0.48	24.08
Metakentrin (Squibb LH)	2	9.36	16.08	0.510	1.89	0.63	20.49
Gonadogen (Upjohn)	4	9.70	16.11	0.337	1.45	1.01	15.27
Chorionic Gonad-otrophin	4	8.30	8.20	0.130	1.71	0.79	12.60
Uninjected Con-trols	3	9.60	8.80	0.135	1.68	0.83	12.31
Normal Controls	6	29.80	52.20	0.852	1.85	0.66	20.94

[1] A relative measure.

creases the proportion of the tubular area and decreases the inter-tubular area, while LH has the opposite effects (table 3).

OTHER GONADOTROPHINS

Both thylakentrin and metakentrin (Squibb) and gonadogen (Upjohn) have the ability of causing testes enlargement and comb growth. It appears that all of these preparations caused the production of androgen (table 3).

Chorionic Gonadotrophin (Upjohn) did not cause any androgen secretion from the testes of hypophysectomized cocks (table 3) and was ineffective in altering either testes weight or the proportion of the various testes components measured.

The dosage of chorionic gonadotrophin used on hypophysectomized cocks (1250 I.U. per bird in eight days) was injected also into two-week old intact male chicks. Here too this hormone preparation

was completely ineffective in either enlarging the comb or increasing testes size. The testes of four control chicks weighed 0.050 grams, while those of the four injected chicks weighed 0.036 grams; the comb areas were 1.80 and 2.06 respectively.

SYNERGISM TRIALS

The results discussed above suggested the possibility that the reason why some "pure" FSH preparations stimulated comb growth while others did not was due to the fact that they were contaminated with quantities of LH too small to be detected by rat assay unless

TABLE 4. EFFECT OF A CONSTANT AMOUNT OF FSH (WIS. 324) MIXED IN VITRO WITH DIFFERENT AMOUNTS OF LH (WIS. P 116BC) ON THE TESTES OF HYPOPHYSECTOMIZED COCKS

Hormones Injected	No. of animals	Comb area Initial	Comb area Final	Testes weight (grams)	Tubule area[1]	Inter-tubular area[1]	Tubule di-ameter[1]
FSH and 0.2 grams equivalent of LH	3	10.08	17.10	0.364	1.61	0.93	15.34
FSH and 0.4 grams equivalent of LH	3	8.40	14.50	0.351	1.44		16.62
FSH and 0.8 grams equivalent of LH	3	9.80	15.80	0.337	1.90	0.63	17.53
Av. of 4 males injected with 5 grams equiv. of FSH 324		9.61	8.90	0.300	2.05	0.76	15.94
Av. of 4 males injected with 5 grams equiv. of LH P116BC		9.30	18.70	0.272	1.41	1.12	14.83
Av. of 2 males injected with 1 gram equiv. of LH P116BC		9.25	9.30	0.125	Not obtained		

[1] A relative measure.

large doses were used. To test this hypothesis a constant quantity of FSH 324, which, when injected alone was unable to cause comb growth, was mixed with a variable amount of LH. In preliminary trials it was found that 1 gram equivalent of LH P 116BC was about the minimum amount of that LH which could cause a detectable comb growth or at least a reddening of the head region of the hypophysectomized cock. On the basis of these results, 5 grams equivalent of FSH 324 was mixed with 0.2, 0.4, and 0.8 grams equivalent of LH P 116BC.

The FSH with the smallest admixture of LH (0.2 grams equivalent) produced as great an effect on comb growth as did the two larger dosages. It is possible that the magnitude of the response produced is inversely proportional to the amount of LH (compare comb areas and testes weights in table 4).

It is also possible that maximal androgen secretion is achieved by the small dose of LH synergizing with FSH, and no further increase in rate of secretion may be produced by increasing the dose of LH.

These tests also established the fact that FSH and LH do produce synergistic effects in the hypophysectomized male. In future experiments the "minimum effective dose" of LH which, when given with FSH, will result in synergistic action is to be determined.

DISCUSSION

On the basis of the information available in the literature it seems that there is some evidence for the possibility that testicular components other than the cells of Leydig secrete androgen. Since it became possible to hypophysectomize large numbers of chickens routinely, it seemed that this animal, by virtue of its prominent and easily measurable head furnishings, would be ideally suited for this kind of study. The comb, wattles, and the ear lobes of the cock regress completely following hypophysectomy (or castration) and may be caused to return to normal only by the administration of androgen. Estrogen in either the male or the female is unable to stimulate or to maintain the head furnishings.

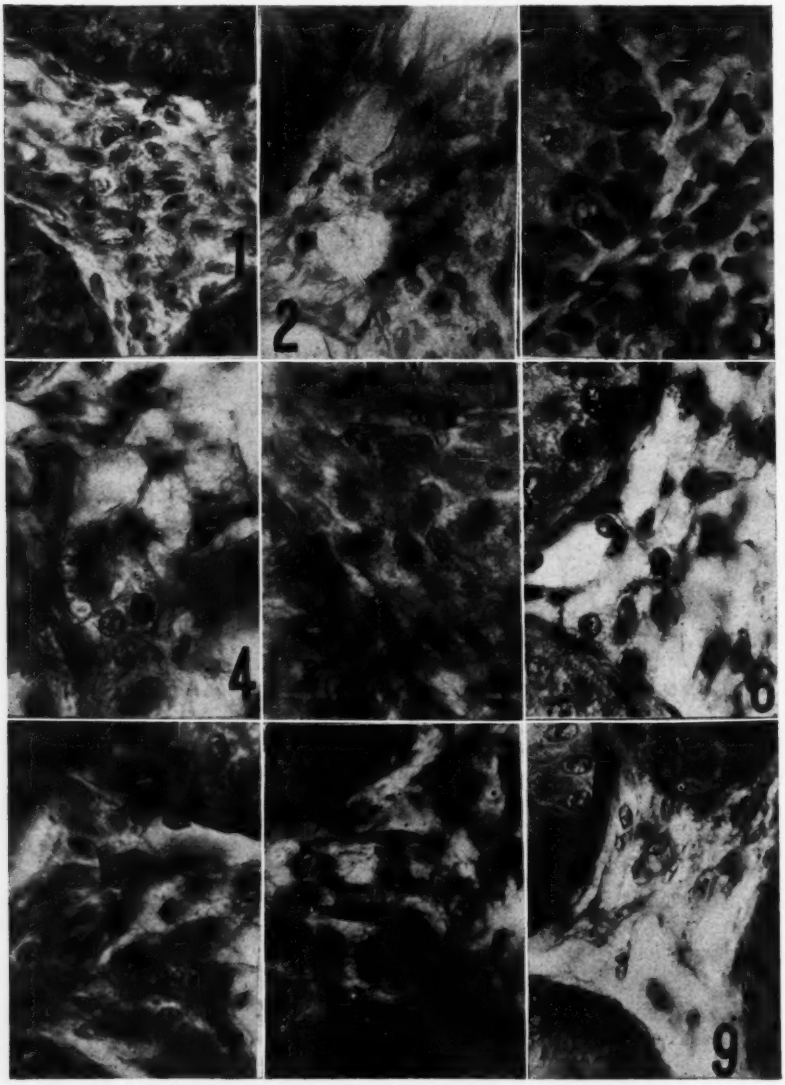
Any increase in the size of the comb and the wattles of a hypophysectomized cock following the administration of a gonadotrophic hormone may be taken as evidence of quantitative and qualitative androgen production. If not enough male sex hormone is present to cause actual enlargement of the comb, the head region will register the presence of traces of androgen by turning red in contrast to the gray pallor commonly seen in the hypophysectomized cock. This fact makes it also possible to eliminate with a great degree of accuracy all incompletely hypophysectomized males and it is not necessary to make microscopic examinations of the sella turcica. The use of large numbers of experimental animals would make this procedure tedious and time consuming.

From the data presented earlier in this paper, the following summary may be made with regard to the observed effects of the gonadotropes used. Comb growth is taken as evidence that the treatment has caused the testes to secrete androgen.

Comb growth resulted from			*Comb growth did not result from*		
FSH	Difco	RX 43732	*FSH*	Wisconsin	324
	Squibb	Thylakentrin			534
	Wisconsin	124			5256
LH	Wisconsin	P 116 BC	*LH*	Difco	RX 43255
		P 325			(Luteogen)
		R 28 A			
	Squibb	Metakentrin			
Other	Wisconsin	F 116 A	*Other*	Upjohn	Chorionic
	Upjohn	Gonadogen			Gonadotrophin

(For the effects of these hormones on testes weight, etc., see Tables 1, and 3.)

If the assumption is granted that all of the above preparations (with the exception of Gonadogen, F 116A, and the Chorionic Gonadotrophin) were, as labeled, free from the other member of the gonadotrophic complex, it would appear that both the FSH and the LH were capable of causing androgen secretion. This would imlpy, according



FIGS. 1 to 9. Effect of various gonadotropes on the testes of hypophysectomized and normal cocks. For details see text.

to the existing theory, that both the Leydig cells (stimulated by LH) and the seminiferous tubules (stimulated by FSH) take part in the elaboration of the male sex hormone.

We knew from rat bio-assays that F 116A contained both LH and FSH, while on the same evidence FSH 124 appeared to be free from LH. Similarly, it was found that small doses of the Difco FSH produced nothing but follicles in the rat, while larger doses produced corpora lutea. No bio-assays were available on thylakentrin; PMS can be regarded as a natural mixture of the two gonadotrophs.

On the basis of these considerations it seemed logical to assume that the ability of some of the FSH preparations used to cause androgen secretion was not due to the FSH itself, but was due to "traces" of LH carried as a contaminant by some of the FSH preparations.

This assumption is further borne out by the fact that deliberate contamination of an FSH which alone cannot induce comb growth with a small amount of LH which, at the level used was also incapable of causing androgen secretion, did cause a pronounced increase in comb size (table 4). What minimum quantities of LH are necessary to produce this synergism with FSH remains to be determined.

The question arises whether LH causes the secretion of androgen by its effect upon the cells of Leydig or upon the germinal epithelium, or a combination of the two effects. This question can be answered, at least in part, by the evidence presented in this paper. Only the Wisconsin preparations of FSH were sufficiently free from LH not to cause comb growth. These FSH preparations were not biologically inactive, as evidenced by the increase in testes weight they produced in the hypophysectomized cock (table 3). They also caused a definite increase in tubule area and some increase in tubule diameter. In spite of this, no determinable amounts of androgen were secreted by the testes, since neither comb growth nor a flushing of the head region were observed.

The question has arisen whether the ability of LH to cause androgen secretion was due to the contamination of the LH with traces of

Fig. no.	Type of male	Treatment (hormone)	Condition of comb
1	hypophysectomized	none	dry
2	hypophysectomized	FSH 324	dry
3	hypophysectomized	Difco "Luteogen"	dry
4	normal	none	large, red
5	hypophysectomized	LH 28A	enlarged, red
6	hypophysectomized	Difco FSH	enlarged, red
7	hypophysectomized	Gonadotrophin 116A	enlarged, red
8	hypophysectomized	Gonadogen	enlarged, red
9	hypophysectomized	Chorionic Gonadotr.	dry

Numbers 1 and 9 show complete absence of vacuolated Leydig cells. Number 2 has a few vacuolated Leydig cells, which in number 3 are not vacuolated but pienotic. Numbers 4, 5, 6, 7, and 8 show typically vacuolated Leydig cells in secretory condition. All magnifications are ×1200.

FSH or whether it was due to the LH itself. Since the tubule diameters of the testes injected with LH were increased, it might be reasoned that the LH preparations were not completely free from FSH. No definite answer can be given to this question at present except that the rat assays indicated the presence of a small amount of FSH. Other experiments to clarify this point are now under way.

The histological findings support the conclusions reached on the basis of the endocrine results. Examination of the intertubular tissue showed that all LH preparations (except Difco LH) caused an increase in the number of Leydig cells and in the proportion of the intertubular connective tissue as a whole. All of the LH and most of the FSH preparations (except Wisconsin FSH 534, 324, and 5256) caused vacuolation of the Leydig cells. The testes of the males treated with Wisconsin FSH showed much intertubular connective tissue, but none of the Leydig cells was vacuolated (Fig. 1).

It is of some interest that the Difco LH is the only one which did not cause comb growth. Rat bio-assays of this preparation showed that it was biologically almost inactive as far as the response of immature ovaries or testes was concerned. It did not synergize with FSH and seemed to have an inhibiting, rather than a stimulating, effect, upon the gonads of the test animals (both rats and chickens). Yet, this preparation was very effective in causing ovulations in normal and FSH pre-treated hens. It was further singular in the respect that it was able to cause oviposition in hens, when hard-shelled, or even soft-shelled, eggs were present in the uterus. Oviposition occurred within a few minutes after this LH was injected intravenously. No pressor substances were found in this preparation (Nalbandov and Card, 1946).

Of some interest is the biologic inactivity of chorionic gonadotrophin in either the hypophysectomized or the immature intact cock. These findings are in accord with the earlier work of Riddle and Polhemus (1931) on doves and pigeons, and many other workers on the intact fowl, pigeon, duck, and English sparrow. In a later publication this failure to cause testicular growth in these animals with PU as ascribed by Lahr, Riddle, and Bates (1941) to the use of insufficiently pure preparations and dosages which were too low. Some of the newer and less toxic preparations used by these authors in dosages from 10 to 5500 RU were highly effective in causing gonadal stimulation. In our work the dosages were certainly sufficiently high (1250 I.U. per bird in 8 days) and the preparation apparently quite nontoxic, but we failed to elicite any response in either the intact immature cock or the hypophysectomized cock. Further work on this subject should settle the question whether the avian testis is capable of responding to PU and whether the bird can be used to differentiate between human chorionic gonadotrophin and PMS.

On the basis of the results presented and discussed in this paper

it is thought that the hypophysectomized cock is ideally suited as a test animal for the following purposes:

a. to determine the purity of FSH preparations. Even slight contamination with LH is detectable and becomes rapidly apparent by causing either a reddening of the head region ("flushing") or, for larger amounts of LH, and therefore androgen, by causing measurable comb growth.

b. to assay the biological activity of FSH. Preparations of FSH free from LH will not cause comb growth but will cause enlargement of the testes. The histological components of the testes also reflect the nature of the gonadotrophic hormone injected.

c. to make comparative studies between the FSH and LH preparations. On the basis of the results presented, it appears possible to differentiate between preparations consisting predominantly of FSH or predominantly of LH. This statement is based on the fact that LH alone will cause both testes enlargement and comb growth, while FSH alone causes only testes enlargement. Preparations consisting predominantly of LH will produce a large comb and testes which are smaller than those produced by preparations containing mainly FSH. More work will be needed before it will be possible to put these relations on a more quantitative basis.

d. to detect small quantities of LH. Amounts of LH which, when given alone are too small to produce a biologically detectable effect, will induce comb growth or at least "flushing" when added to an FSH preparation which alone cannot cause these effects.

SUMMARY

Using hypophysectomized cocks as test animals, it has been found that preparations of FSH absolutely free from LH will not cause enlargement of the capon-type comb of the completely hypophysectomized cock. Such an FSH will cause increase in testes size.

LH alone causes both comb growth and enlargement of the testes.

FSH contaminated with even small amounts of LH will cause both comb growth and increase in testes weight. There is a synergistic effect between these two hormones.

It is suggested that the hypophysectomized cock is well suited to serve as a test animal for the determination of the purity of FSH preparations. Even small amounts of LH, when admixed to an FSH preparation, can be detected by the disappearance of the characteristic pallor of the head region of the hypophysectomized cock. Larger amounts of LH will be detected by measurable increases in comb size.

The results obtained further increase the evidence in favor of the Leydig cells as the source of androgen.

Large doses of chorionic gonadotrophin, contrary to most recent work but in agreement with earlier studies, do not have any measur-

able biological effect in either the immature intact chick or the hypophysectomized cock.

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THE EXPERIMENTAL PRODUCTION OF MENSTRUAL ANOMALIES[1,2]

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THE PURPOSE of this report is to present certain experimental observations bearing on the problem of functional menstrual disorders. Our first approach to this problem was through a study of the endometrial changes associated with abnormal uterine bleeding and the production of comparable changes in experimental animals. By correlating experimental results with clinical findings, we have been able to arrive at an understanding of the etiology of functional menstrual disorders. This may be stated as follows.

Functional menstrual disorders may result from lesions in any of the endocrine glands or from general constitutional disease secondarily affecting the endocrine system. The primary disease condition gives rise to ovarian hypofunction and this in turn gives rise to the menstrual abnormality. There is no constant correlation between the primary lesion, the degree of ovarian involvement as indicated by the endometrial histology and the type of menstrual disorder (Burch, 1936; Burch and collaborators, 1937).

This concept correlated what was known concerning female reproductive physiology and provided for the first time a rational basis for the treatment of menstrual disorders. It indicated the prevalence of early stages of endocrine disease and emphasized the need for more adequate methods of diagnosis and treatment. Much of the current research in the field of Endocrinology is directed toward this end.

Our second approach to the problem of menstrual disorders has been through a study of the local vascular mechanisms involved in the production of the bleeding abnormalities. The full significance of this phase of the investigation can best be appreciated when viewed against the background of previous work. However, this has been reviewed elsewhere (Burch and Phelps, 1942, 1943) and it is necessary to recall here only the endometrial changes which result from ovarian hypofunction and their relationship to the menstrual symptoms.

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[2] Hormone preparations used were Progynon B and Proluton, supplied by the Schering Corporation through the courtesy of Dr. Erwin Schwenk, and progesterone in sesame oil supplied by the S. E. Massengill Company.

Ovarian failure is a gradual process, affecting first the postovulatory phase of the cycle, progressing to complete absence of the postovulatory phase and finally to the postmenstrual level or lower. Three progressively more severe levels of ovarian hypofunction can be distinguished on the basis of the resulting endometrial changes. These we have designated, in the order of increasing severity, first—characterized by abnormalities of the corpus luteum, with, however, definite progestational changes in the endometrium, second—characterized by absence of the corpus luteum, hence an unmodified estrogenic reaction in the endometrium, and third—characterized by a subnormal estrogenic reaction in the endometrium.

Within each of these categories, there are variations in the intensity of the characteristic endometrial reaction, indicating variations in the level of ovarian activity. Stages transitional between one level and the next have been seen frequently. These are indicated by a merging of the histologic features definitive of the one level with those definitive of the other.

In correlating the menstrual abnormalities with the endometrial histology, it has been found that no single symptom is characteristic of any degree of ovarian failure, although there is a tendency for the minor disturbances of interval and flow to fall in the first group, the severe bleeding in the second group, the amenorrheas in the third.

The lack of parallelism between the degree of ovarian hypofunction and the type of menstrual abnormality indicates that there is involved in the production of the menstrual symptoms a mechanism, presumably vascular, which is not reflected in the endometrial histology, or, if reflected there, is not recognizable in the present state of knowledge. Therefore, the important question of the relationship of the bleeding abnormalities to ovarian failure has been approached through a study of the endometrial vascular bed.

Previously, Doctor Rucker Cleveland (1941), then of the Department of Anatomy, had studied the effects of various hormone combinations upon the endometrial histology in monkeys and she had noted that bleeding was prolonged in three of five experiments in which a certain combination of estrogen and progesterone was given.

In the present investigation, castrated monkeys had been injected with this and other combinations of estrogen and progesterone and with estrogen alone. The effects on vaginal bleeding have been noted and the effects on the endometrial vascular bed studied by means of intraocular endometrial transplants (Markee, 1940). Seventy-three experiments have been performed in nine monkeys, six of which had also been used in Cleveland's (1941) study.[3]

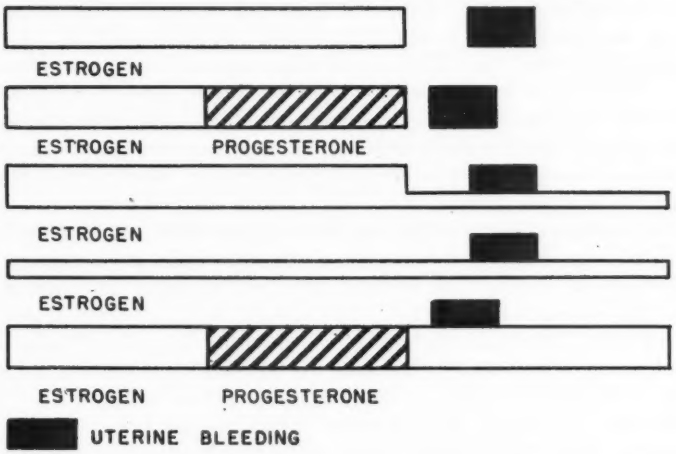
[3] The histologic responses indicated in the tables were demonstrated in previous studies (Cleveland, 1941; Cleveland, Phelps and Burch, 1941). For the sake of completeness, results obtained with estrogen in these previous studies are included in the data on vaginal bleeding (12 experiments in group I, 6 experiments in group II).

The experimental production of the vascular changes characteristic of the normal menstrual cycle has been reported recently (Phelps, 1946). An action of progesterone upon the endometrial vascular bed was demonstrated and the significance of these observations in relation to implantation of the fertilized ovum indicated. The present report is concerned with observations on vaginal bleeding.

The findings of many investigators who have studied the hormonal control of menstruation are illustrated in chart 1. They may

CHART I

EXPERIMENTAL EVIDENCE CONCERNING THE HORMONAL CONTROL OF UTERINE BLEEDING



be summarized as follows. Removal of the ovaries is followed by a premature menstrual period. In castrated females, bleeding follows cessation of treatment with either or both ovarian hormones. It does not occur during treatment with sufficiently large dosages, but may occur following sudden reduction of the daily dose below a certain critical level, during prolonged treatment with a daily dosage that approximates the critical level or during treatment with larger doses of estrogen if progesterone is introduced and withdrawn. The bleeding produced by these types of treatment is similar to normal menstruation in amount, duration and local vascular phenomena (Allen, Danforth and Doisy, 1939; Corner, 1939; Markee, 1940).

The following generally accepted conclusions have been drawn from this and other evidence of similar import: 1) the endometrium does not bleed as long as an adequate level of stimulation is maintained and 2) the endometrium does bleed when deprived of stimula-

tion and some form of hormone deprivation is an essential prerequisite for bleeding.

The so-called threshold level, or dose, may be defined as the maximum daily dose that will not prevent bleeding following reduction of the dosage, when only estrogen is given (Zuckerman, 1936, 1937; Corner, 1938). It varies within narrow limits. The bleeding that occurs during treatment with the threshold dose is considered a special type of hormone deprivation bleeding, the deprivation being accomplished by fluctuations in the sensitivity of the body to estrogen rather than by fluctuations in the amount of hormone available. Zuckerman (1937 b) has shown that the average threshold dose for the rhesus monkey is about 100 international units daily, the range of variation from about 50 to 300 international units daily.

In the present study, all of the types of hormone deprivation indicated in chart 1 have been employed. High, moderate and low dosages have been used. The low dosages approximate the threshold level. Moderate dosages are within the range known to produce more or less normal histologic responses. The high level employs a dosage of estrogen with which we have produced glandular cystic hyperplasia in castrated macaques (Cleveland, Phelps and Burch, 1941.)

The monkeys were inspected for vaginal bleeding at least once daily. All bleeding recorded was of macroscopic proportions, i.e., comparable in amount to clinical uterine bleeding. In episodes of normal to subnormal duration, the amount of flow varied from normal to profuse. In prolonged episodes, the daily loss varied from moderate to very slight and there was no constant correlation between dosage and amount of flow.

Bleeding of longer than 8 days' duration has been classified as prolonged. This is somewhat arbitrary, but seems justified on the basis of abundant pertinent data in the literature (reviewed by Cleveland, 1941 b).

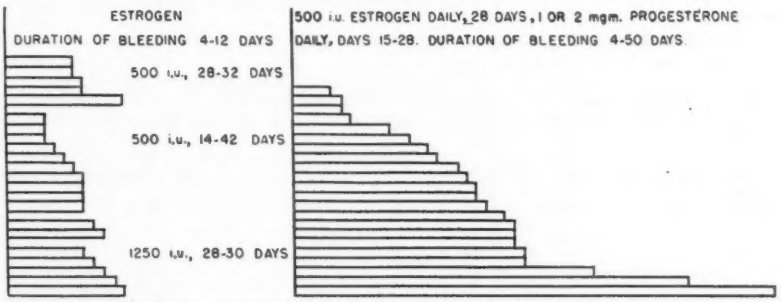
For the purposes of the present report, the experiments have been classified in four groups on the basis of relative level of stimulation and type of hormone deprivation used to precipitate bleeding.

GROUP I

The dosage, method of administration and results of the 65 experiments in this group are shown graphically in charts 2 and 3 and summarized in table 1. The significance of these results is three-fold. Note first the production of menorrhagia. Bleeding of 13 to 50 days' duration was produced in 22 instances, mild prolongation of bleeding in 13 instances. The second feature of significance is the lack of correlation between stimulus, histology and duration of bleeding, i.e., whether normal or prolonged. Prolongation was produced with estrogen-progesterone and with estrogen alone. Each dosage that produced

CHART 2

DURATION OF VAGINAL BLEEDING, EXPERIMENTS OF GROUP I


ESTROGEN
DURATION OF BLEEDING 4-12 DAYS
500 i.u., 28-32 DAYS
500 i.u., 14-42 DAYS
1250 i.u., 28-30 DAYS
500 i.u. ESTROGEN DAILY, 28 DAYS, 1 OR 2 mgm. PROGESTERONE DAILY, DAYS 15-28. DURATION OF BLEEDING 4-50 DAYS.
EACH BAR REPRESENTS RESULTS OF ONE EXPERIMENT, DURATION OF VAGINAL BLEEDING IN DAYS

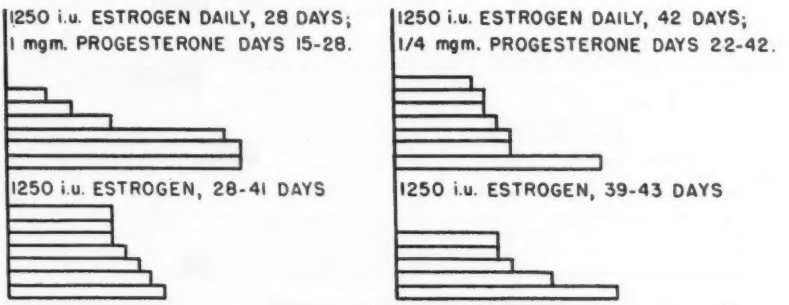
Experiments of group I. Duration of macroscopic vaginal bleeding produced by cessation of treatment with moderate daily dosages of the ovarian hormones.

prolongation also produced bleeding of normal duration. Bleeding of normal and abnormal duration took place from a progestational endometrium and from a proliferative endometrium. Finally, there are the differences and similarities in the various dosages with respect to the frequency with which prolongation was produced and maximum duration of the prolonged episodes produced. This feature will be discussed later.

CHART 3

DURATION OF VAGINAL BLEEDING

EXPERIMENTS OF GROUP I, CONTINUED


1250 i.u. ESTROGEN DAILY, 28 DAYS; 1 mgm. PROGESTERONE DAYS 15-28.
1250 i.u. ESTROGEN, 28-41 DAYS
1250 i.u. ESTROGEN DAILY, 42 DAYS; 1/4 mgm. PROGESTERONE DAYS 22-42.
1250 i.u. ESTROGEN, 39-43 DAYS

DURATION OF BLEEDING

ESTROGEN-PROGESTERONE:	3-18 DAYS	ESTROGEN-PROGESTERONE:	6-16 DAYS
ESTROGEN :	8-12 DAYS	ESTROGEN :	8-17 DAYS

Experiments of group I, continued. Duration of macroscopic vaginal bleeding produced by cessation of treatment with moderate daily dosages of the ovarian hormones.

TABLE 1
Group I

Relative level of stimulation moderate, bleeding precipitated by cessation of treatment.

Daily dose		Number of episodes of bleeding	Duration of bleeding, days	Number of episodes of duration indicated				Endometrial reaction
Estrogen I.U.	Progesterone, mg.			3–8	9–12	13–18	19–50	
500	1 or 2	22	4–50	4	2	4	12	progestational
1250	1	6	3–18	3		3		
1250	¼	7	6–16	4	2	1		
500		5	7–12	4	1			proliferative
		12	4–10	10	2			proliferative
1250		13	7–18	5	6	2		proliferative

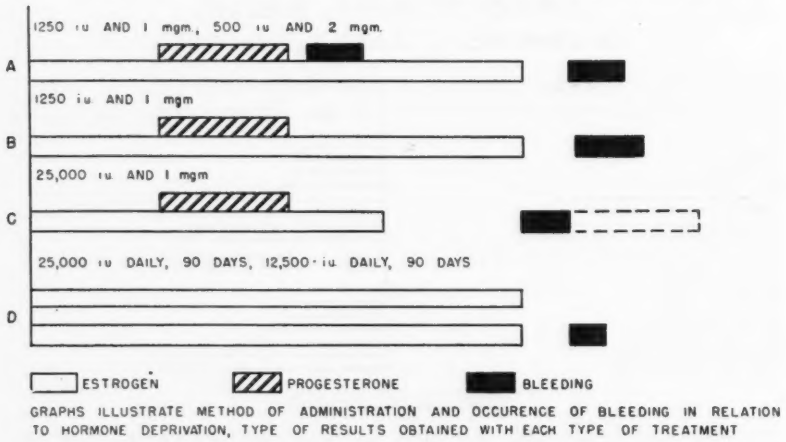
GROUP II

The results of the 15 experiments in this group are presented in chart 4 and table 2.

Subgroups A, B, C, chart 4. In these experiments, estrogen and progesterone were administered as in the experiments of group I, but only the progesterone was discontinued at the end of the estrogen-progesterone phase. Progesterone withdrawal was usually followed by bleeding, but not always.

CHART 4

TREATMENT AND TYPE OF RESULTS IN EXPERIMENTS OF GROUP II


1250 iu AND 1 mgm., 500 iu AND 2 mgm.
A
1250 iu AND 1 mgm
B
25,000 iu AND 1 mgm
C
25,000 iu DAILY, 90 DAYS, 12,500 iu DAILY, 90 DAYS
D
ESTROGEN
PROGESTERONE
BLEEDING
GRAPHS ILLUSTRATE METHOD OF ADMINISTRATION AND OCCURENCE OF BLEEDING IN RELATION TO HORMONE DEPRIVATION, TYPE OF RESULTS OBTAINED WITH EACH TYPE OF TREATMENT

Experiments of group II. Types of treatment and types of results obtained.

Estrogen was continued, with no change in dosage, for 25 days or longer after progesterone withdrawal in experiments of types A and B and for a maximum of 10 days in experiments of type C.

In the two experiments of group C, bleeding did not occur following progesterone withdrawal and the latent period following estrogen withdrawal was unusually prolonged. The bleeding was of normal duration in one case, normal followed by very slight spotting in the other.

Subgroup D, chart 4. With the treatment indicated, the usual result was no bleeding at all. However, bleeding did occur in one experiment.

The failure of bleeding to occur following hormone deprivation may be considered a type of amenorrhea. And it may be noted in pass-

TABLE 2

Group II

Relative level of stimulation high or moderate, bleeding precipitated by hormone deprivation; a) progesterone discontinued, estrogen continued at the same level or b) estrogen discontinued.

Daily dose		Number of instances of no bleeding	Number of episodes of bleeding	Duration of bleeding, days	Endometrial reaction
Estrogen I.U.	Progesterone, mg.				
25,000	1	2			
1250	1	1	4	6–8	
500	2		1	5	progestational
25,000 prolonged		6	1	4	proliferative or hyperplastic
25,000 short			2	4, 19	

ing that failure of bleeding to occur during prolonged treatment with one or both ovarian hormones (e.g., subgroup D, chart 4) is also comparable to clinical amenorrhea.

Reference to table 2 reveals that amenorrhea was produced with estrogen-progesterone and with estrogen. Certain dosages which produced amenorrhea also produced bleeding of normal duration.

Results similar to those obtained in the experiments of group II have been reported previously (e.g., Zuckerman, 1937 c) but the implications in relation to ovarian failure have not been emphasized.

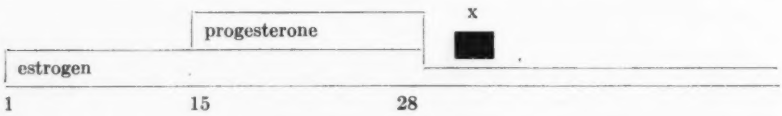
GROUPS III A AND III B

In the experiments of group III, the two hormones were injected as before, up to the end of the estrogen-progesterone phase. Then the daily dosage was reduced to the threshold level. In some instances, estrogen only was injected during the low-dosage phase, in others, both estrogen and progesterone. In all 10 of the experiments of this type, bleeding occurred following reduction of the dosage. Nine episodes were of normal duration, one was moderately prolonged (table 3).

TABLE 3
Group III A

Relative level of stimulation moderate, bleeding precipitated by hormone deprivation: a) progesterone discontinued, estrogen continued at a reduced level or b) daily dosage of both hormones reduced simultaneously.

Daily dose		Number of episodes of bleeding	Duration of bleeding, days	Endometrial reaction
Estrogen I.U.	Progesterone, mg.			
500	2	2	3, 18	progestational
1250	1	7	3–8	
1250	¼	1	4	



Following reduction of the dosage, treatment at the threshold level was continued for periods varying in duration from 21 to 148 days. The results obtained during the low-dosage phase are presented in charts 5 and 6 and table 4. Chart 5 shows the results obtained when only estrogen was given during the low-dosage phase. Note the periodic occurrence of bleeding, the occasional prolonged episodes and the one instance in which bleeding failed to occur following cessation of treatment. Note also the occurrence of bleeding during periods of no treatment.

Chart 6 shows the results obtained when both estrogen and progesterone were given during the low-dosage phase. Again, successive episodes of bleeding occurred, the duration was normal to subnormal, with one episode slightly prolonged, and intervals between episodes varied in duration from subnormal to markedly prolonged. Bleeding failed to occur following cessation of treatment in one instance.

Thus, with the low dosages (table 4), irregularities of the interval

TABLE 4
Group III B

Relative level of stimulation low, bleeding occurred during prolonged treatment, with or without any change in daily dosage.

Daily dose		Number of of episodes of bleeding	Duration of bleeding, days	Number or prolonged episodes	Duration of interval, days	Number of instances of no bleeding after last injection
Estrogen I.U.	Progesterone, mg.					
125	¼	15	1–13	1	15–125	1
125		10	1–43	3	21–46	1
none	none	2	2		42–115*	

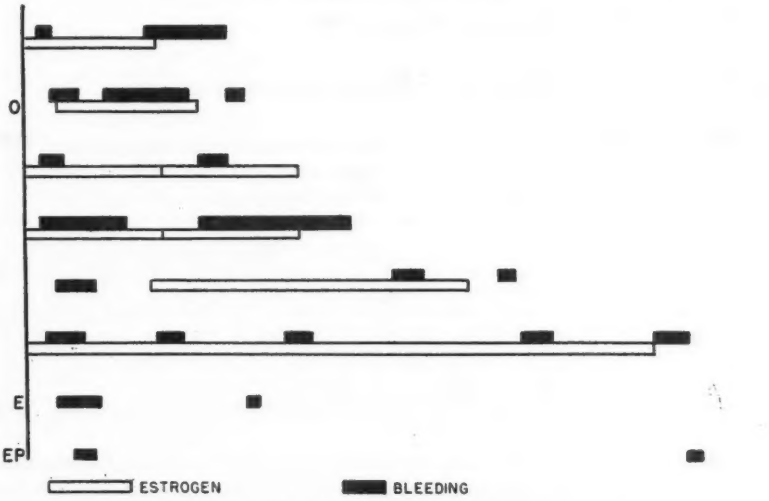
* Last injection to onset of bleeding.

and flow were produced. Duration of bleeding varied from 1 to 43 days, duration of the intervals between episodes from 15 to 125 days.

It will be noted that anomalies comparable to all of the basic types of clinical abnormalities of the menstrual interval and flow have been produced and likewise results comparable to clinically normal

CHART 5

EXPERIMENTS OF GROUP III B



Experiments of group III B. Experiments of group III in which only estrogen was given during the low-dosage phase.

Each graph begins with the day dose was lowered. The first episode of bleeding shown is the one that resulted from lowering of the dosage (x, table 3). The rest of the graph shows the effect of prolonged treatment at the threshold level.

Graph O. Castration at O, treatment with estrogen begun after bleeding had started.

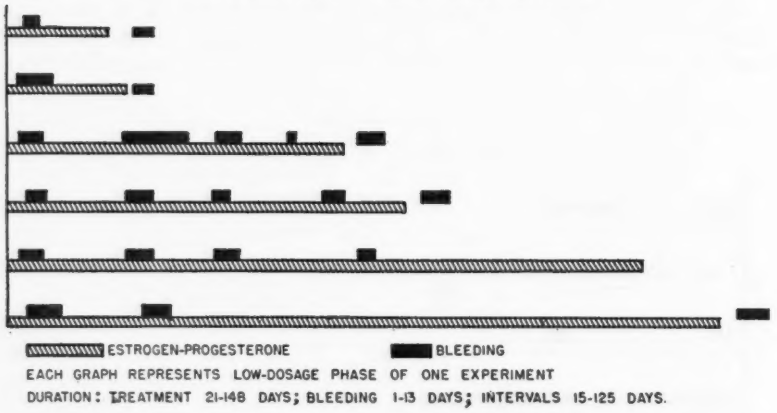
Graph E. Experiment of group I. Estrogen discontinued at E, no further treatment. Second episode of bleeding began 42 days after the last injection.

Graph EP. Experiment of group I. Estrogen-progesterone discontinued at EP, no further treatment. Second episode of bleeding began 115 days after the last injection.

menstruation: bleeding of normal duration, bleeding of subnormal duration, menorrhagia, intervals of normal duration, intervals of subnormal duration and three types of amenorrhea, namely, prolonged intervals between episodes, failure of bleeding to occur in certain instances of hormone deprivation and failure of bleeding to occur during prolonged treatment with moderate and large dosages. Each type of anomaly was produced with estrogen and also with

CHART 6

EXPERIMENTS OF GROUP III B, CONTINUED


ESTROGEN-PROGESTERONE
BLEEDING
EACH GRAPH REPRESENTS LOW-DOSAGE PHASE OF ONE EXPERIMENT
DURATION: TREATMENT 21-148 DAYS; BLEEDING 1-13 DAYS; INTERVALS 15-125 DAYS.

Experiments of group III B, continued. Experiments of group III in which both estrogen and progesterone were given during the low-dosage phase.

Each graph begins with the day dose was lowered. The first episode of bleeding shown is the one that resulted from lowering the dosage (x, table 3). The rest of the graph shows the effect of prolonged treatment at the threshold level.

estrogen-progesterone and, in each category, with dosages varying considerably in magnitude (table 5).

The treatment in these experiments cannot be compared quantitatively with clinical ovarian failure because we do not know the quantitative hormonal output of the ovaries in either the normal or abnormal state. However, on the basis of hormones available, known effects on endometrial histology and relative strength of the dosages as compared with each other, the various dosages can be classified in four categories corresponding, respectively, to the three degrees of ovarian failure defined above and levels transitional between the first and second degrees.

TABLE 5. RELATIVE LEVEL OF STIMULATION AND TYPE OF RESULTS AS COMPARED WITH OVARIAN FAILURE

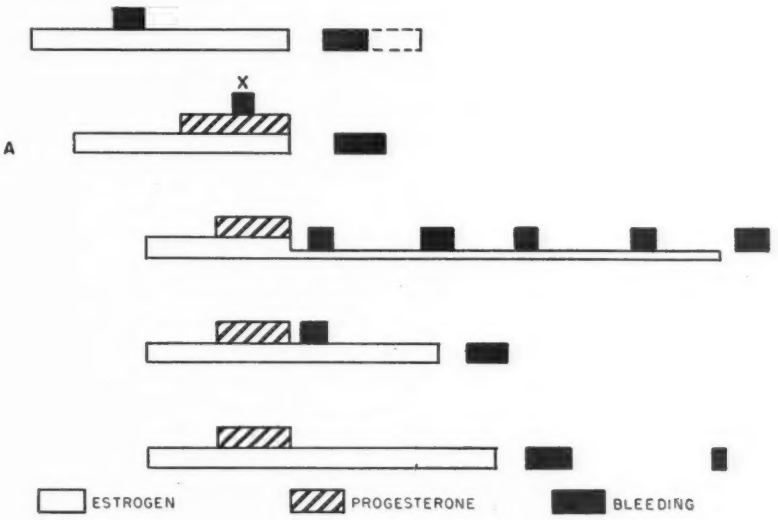
Relative level as compared with ovarian failure	Number of episodes of bleeding	Number of episodes of duration indicated				Number of intervals of duration indicated			Number of instances of no bleeding after hormone deprivation
		4–8	1–3	9–18	19–50	26–31	15–24	42–125	
1st degree: 500 & 1 or 2 1250 & 1	48	18	7	11	12				1
1st–2nd degree: 1250 & 1 25,000 & 1	12	6	2	4					2
2nd degree: 25,000; 1250; 500	20	9	2	9					6
3rd degree: low dosages; no treatment	27	17	6	2	2	4	18	6	2
Totals	107	50	17	26	14	4	18	6	11

Reference to table 5 reveals that there was no constant correlation between the components of the stimulus and the type of menstrual symptom produced and no constant correlation between the type of menstrual symptom and the relative level of stimulation as compared with ovarian failure.

These results provide an important link in the chain of evidence supporting the ovarian failure concept stated above. They afford experimental evidence of a cause and effect relationship between menstrual abnormalities and ovarian function and confirm the clinical

CHART 7

OCCURENCE OF BLEEDING IN RELATION TO CHANGES IN COMPONENTS AND RELATIVE STRENGTH OF STIMULUS



evidence that there is involved in the production of these disorders a mechanism primarily vascular which is not dependent upon the endometrial histology. They confirm the clinical observation that there is no constant correlation between the type of menstrual abnormality and the level of ovarian activity, thus emphasizing the unreliability of uterine bleeding as an indicator of ovarian function. The implications relative to normal menstruation are especially important. It is clear that a distinction should be made between normal menstruation and menstruation of normal duration. Menstruation of normal duration, whether occurring spontaneously or in response to therapy, does not necessarily indicate normal ovarian function.

Results of the type illustrated in chart 7 further support the statement just made. In five instances, bleeding occurred during

continuous treatment with dosages that are well above the bleeding threshold of the rhesus monkey—twice during treatment with 1250 I.U. estrogen plus ¼ mgm. progesterone, twice during treatment with 1250 I.U. of estrogen and once during treatment with 25,000 I.U. of estrogen. In a sixth experiment, in which the daily dosage was 500 I.U. estrogen plus 2 mgm. progesterone, bleeding from the intraocular transplant occurred at the stage indicated by x in graph A, chart 7, but no vaginal bleeding was seen. Duration of these episodes was 1–5 days, i.e., subnormal to normal. This was true functional bleeding in

TABLE 6. OCCURRENCE AND DURATION OF BLEEDING IN RELATION TO COMPONENTS, RELATIVE LEVEL AND DURATION OF THE CURRENT HORMONAL STIMULUS

Daily dose		Number of episodes of bleeding*	Duration of bleeding, days	Number of prolonged episodes	Number of instances of no bleeding after hormone deprivation†	Duration of intervals between episodes, days
Estrogen I.U.	Progesterone, mg.					
500	2	16	4–50	13		
500	1	6	6–23	5		
1250	1	6	3–18	3	1	
1250	¼	7	6–16	3		
125	¼	15	1–13	1	1	15–125
500/7–21 days		7	2–6			
28–42 days		11	4–12	3		
1250/28–41 days		7	8–12	4		
43–76 days		6	7–18	2		
125		10	1–43	3	1	21–46
25,000	1	2	4, 19	1	2	
25,000		1	4		6	

* Includes only episodes precipitated by cessation of treatment.
† Includes experiments of groups II and III.

the sense that it resulted from vascular changes induced by hormonal stimulation and essentially normal in character. Some of these vascular changes were described in a previous report (Phelps, 1946).

These results, while few in number, are of considerable importance, for they indicate that the endometrium can bleed without hormone deprivation in the generally accepted sense. They suggest, therefore, that hormone deprivation may not be an essential prerequisite for abnormal bleeding.

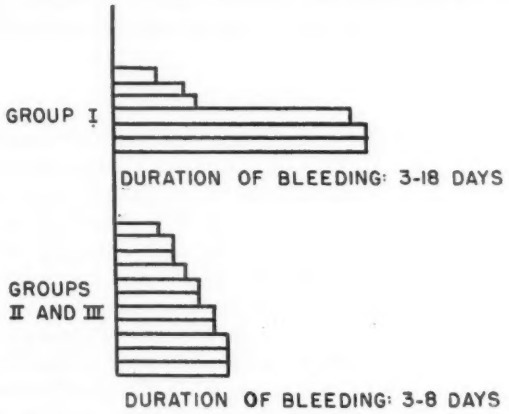
For the rest, chart 7 illustrates the observations on occurrence of bleeding in relation to the level of stimulation and fluctuations in same. Bleeding occurred in all possible relationships: following various types of hormone deprivation, during continuous treatment at

high and moderate levels, during continuous treatment at a low level and occasionally during periods of no treatment. These observations likewise emphasize the unreliability of uterine bleeding as a diagnostic criterion and indicate the need for further studies bearing on the significance of uterine bleeding in terms of ovarian activity.

Comparison of the various dosages employed, with respect to the type of results produced, the relative frequency with which anomalies

CHART 8

DURATION OF BLEEDING PRODUCED WITH 1250 i.u. ESTROGEN AND 1 mgm. PROGESTERONE



Duration of macroscopic vaginal bleeding in all experiments in which the dosage indicated was administered. In the experiments of Group I, bleeding was precipitated by cessation of treatment. In the experiments of groups II and III, bleeding was precipitated by progesterone withdrawal or by decreasing the daily dosage to the threshold level.

were produced and the maximum duration of the bleeding produced (table 6) indicates that the duration of any given episode of bleeding is influenced by the components and relative strength of the stimulus that produced it.

The variations in the effects of each dosage (table 6) indicate that the duration of any given episode is also influenced by something else. In other words, the stimulus that produced the bleeding is not the only influence involved. For example, note the wide range of variation in the results obtained with 500 I.U. of estrogen plus 1 or 2 mgm. progesterone (table 6). Since no further treatment was given after hormone withdrawal, it is immediately apparent from the graph of the results (chart 2) that the duration of bleeding must have been influenced by something in the previous history of the animal.

Consider now the 17 episodes of bleeding produced with 1250 I.U. of estrogen plus 1 mg. progesterone (chart 8). These results suggest that bleeding is influenced by stimuli acting subsequent to the onset of flow.

The observations presented support the conclusion that uterine bleeding is not controlled by any one hormonal influence but by a combination of influences. The nature of any given episode of bleeding is probably dependent upon the interaction of the factors involved. The evidence at hand indicates that these factors fall into three categories: 1) the current hormonal stimulus, i.e., the one that produced the bleeding, 2) conditions existing prior to application of the current stimulus and 3) stimuli acting subsequent to the onset of flow. Evidence concerning the nature of each of these three classes of influence and the implications for further research will be presented in subsequent reports.

SUMMARY

Castrated macaques were injected with various combinations of the ovarian hormones. Menstrual anomalies comparable to all of the basic types of clinical abnormalities of menstrual interval and flow were produced. Each type of anomaly was produced with estrogen and with estrogen-progesterone and, in each category, with dosages varying considerably in magnitude. There was no constant correlation between type of menstrual symptom and relative level of stimulation as compared with ovarian failure. Bleeding occurred in all possible relations to the level of stimulation and fluctuations in same. The results afford evidence of a cause and effect relationship between menstrual abnormalities and ovarian function, confirm the clinical evidence that there is involved in the production of these disorders a mechanism primarily vascular which is not dependent upon the endometrial histology, emphasize the unreliability of uterine bleeding as an indicator of ovarian function and support the conclusion that uterine bleeding is not controlled by any one hormonal influence but by a combination of influences. The evidence presented indicates three classes of influence: 1) the current hormonal stimulus, 2) conditions existing prior to application of the current stimulus and 3) stimuli acting subsequent to the onset of flow.

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THE WEANLING GUINEA PIG AS TEST OBJECT FOR ESTROGEN ASSAYS

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SINCE ALLEN and Doisy in 1923 published their useful test for "follicular hormone" other more delicate assay methods have been developed. Lyons and Templeton (1936) showed that by instillation of the material to be tested into the vaginal lumen (and hence in immediate contact with the vaginal wall) a much smaller amount of estrogen could be detected. The endpoint in their experiments was frank cornification, as in the Allen-Doisy test, whereas Fluhmann (1933, 1934) originated an even more sensitive test by taking advantage of the mucification of the superficial cells of the vagina in the castrated mouse, a transformation which requires much less estrogen than is needed for cornification. Albrieux (1936) went further when he combined both methods and added a device of his own for assaying blood estrogen. He first dried a column of blood in a collodion "straw," then inserted a small "pellet" of the dried blood into the vagina of the spayed mouse. Mucification was the endpoint in this test. Markee and Berg (1944) utilized some of the preceding methods to assay differences in the estrogen titer of blood of women on the various days of the menstrual cycle. The endpoint was the cell and mucus content of material sucked up into a capillary pipette from the vagina.

The methods to date, although very delicate, are more or less laborious. The blood must be processed and the mice castrated and standardized. To obviate this difficulty the writers reported (1945) a method which has proved delicate enough to detect the estrogenic hormones in a small drop of untreated blood fresh from a woman's finger or ear-lobe. The method has the added advantage that not only do the experimental animals (infantile rats, mice or guinea pigs) not have to be prepared—they need not even be sacrificed, but, after the observation has been made on them, they may be returned to the colony.

The new method depends upon the opening of the vagina of the immature rodent, a phenomenon already known to Allen and Doisy (1923), who injected the material to be tested subcutaneously for absorption into the systemic circulation. In the present method the

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injection is made in the region of the vaginal orifice, as briefly described by us (*Science*, August 17, 1945). A short-beveled needle is inserted just beneath the skin, as shown in figure 1. The response has always been positive with 0.02 cc. of woman's blood, always negative to blood from men.

While the immature rat and mouse constitute favorable material for this test the present writers felt that, because of certain peculiari-

FIG. 1. Illustrating method of injecting 21-day-old female rat (cf. *Science*, August 17, 1945.)

ties of the guinea pig, this species might prove superior to the Muridae for the test. The present paper reports the results of some experiments on this species.

USE OF THE GUINEA PIG FOR ESTROGEN ASSAY

It is well known from the work of Stockard and Papanicolaou (1917), Kelly and Papanicolaou (1927) and Kelly (1929) and others that the closure membrane of the guinea pig vagina disappears with the approach of heat, to be regenerated after four days and to remain intact for the remaining twelve days of the sixteen-day cycle. In common with other rodents the vagina of the juvenile female remains closed until the approach of the first heat period and it also remains closed during pregnancy and after castration in the adult.

In this paper the writers report their experiments with known

quantities of estrogen. Adult females were tried in an exploratory manner, but were soon discarded in favor of juveniles (weanlings) weighing around 200 grams. These were found to be as sensitive as the sixteen or the twenty-one-day rat. Castrated adults have also been found extremely sensitive, especially when first primed a week before use; but these cases are not considered further in this paper.

FIG. 2. Illustrating method of injecting young female guinea pig (see text).

TECHNIQUE OF INJECTION

The total dosage employed was administered in two injections, one-half on either side of the closure membrane. As shown in figure 2, the needle is inserted at the tip of each horn of the crescentic depression extending across the perineum, and is directed towards the midline underneath the horn of the crescent. Since it is important that the injection be made with precision, a second operator is essential in order throughly to immobilize the animal (figure 2).

ENDPOINT TERMINOLOGY

When the closure membrane is intact the vaginal area is dry and pallid. Within a very few hours after the injection of oil or blood containing estrogen, the vulvar and circumanal regions appear conjested,

an effect presumably due to the hormone, since oil without hormone does not produce this effect and with male blood the congestion is slight.

If observations are made at close intervals, preferably hourly, five stages of the reaction may be distinguished:

Line formation. This consists of an intense blanching, somewhat

FIG. 3. Perineum of young female guinea pig, showing intact closure membrane and crescentic area.

like the stigma of a maturing egg follicle in the hen. It usually forms in the mid-portion of the vaginal crescent.

Dimpling. This appears as an indentation much like the infolding of the dorsal lip of the blastopore of the frog egg. Since line formation may be quite transitory, dimpling often seems to be the first stage in the reaction. It is followed by either stage 3 or stage 4.

Pinpoint formation. A pinpoint opening may occur at any place in the vaginal crescent. Although very small, it is a definite opening into which an applicator may be inserted. It may increase in size and become a slit or remain a pinpoint opening for several hours. Often the entire crescent may then dimple and complete opening follow.

Slit formation. A slit-like break may follow line or pinpoint formation. The slit may be present from 3 to 18 hours before the vagina opens completely.

Complete opening, with secretion of mucus and cornification of the mucosa (figure 4).

Lest it seem over-meticulous to differentiate between these stages it should be said that this is necesssary because the process of opening may be arrested at one of these stages whenever the quantity of the injected hormone is insufficient to carry the process to completion.

FIG. 4. Showing open vagina of treated young guinea pig. The vaginal opening has been spread by applying tension ventrally.

Mucus may issue from the slightest opening in the closure membrane, moistening the region externally.

MATURE ADULTS

Since the closure membrane of the adult cyclic female guinea pig remains intact for 12 out of the 16 days of the normal estrous cycle, it would seem reasonable to suppose that such a female might be used for hormonal assay after the method described, as soon as the membrane has regenerated. This hypothesis was investigated.

In order to establish their normality the animals used (chart 1) were observed daily through 4 complete cycles before any experiments

were attempted. The average length of the cycles of 11 females was 16.3 days, the period of opening was on the average 3.5 days in length. The preliminary cycles are not registered in the chart.

In the initial experiments injections were made on the fourth day (indicated by carets in chart 1) after regeneration of the closure plate; but this choice of day proved unsatisfactory, since no significant shortening of the "closed" period was effected. It is possible

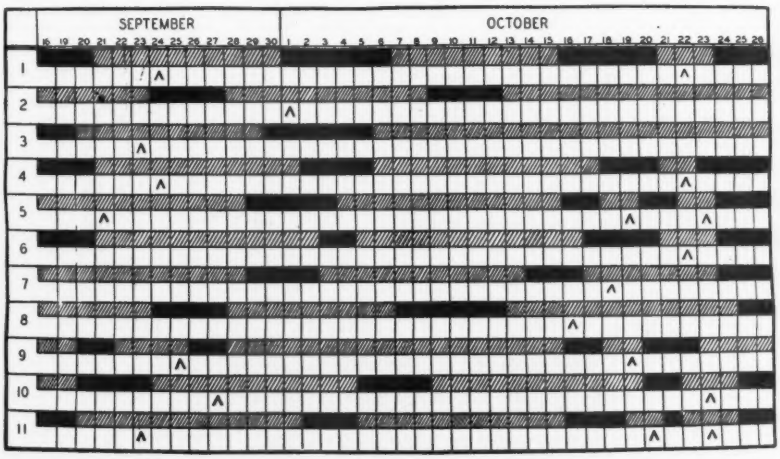

CHART 1. Showing time vaginas of 11 adult females remained open (solid areas) or closed (cross-hatched areas). Carets indicate injection of woman's blood.

that the progesterone of the animals' corpora lutea complicated the picture.

After this preliminary experiment, cycles were again established (chart 1) and blood was injected on the first day of the closure. It is seen from the chart ("October 16–23") that, in most cases, the vagina opened on the first or the second day following the injections.

The quantity of blood used varied from 0.02 to 0.08 cc.

THE IMMATURE GUINEA PIG

The results of injecting the immature females are shown in table 1 and chart 2. It is seen that opening of the vagina is effected promptly as rather large doses, 0.05 to 0.08 cc. were employed—doses unnecessarily large, as later discovered. Animals 30, 36 and 37 showed only pinpoint openings within 24 hours and No. 34 required 48 hours to open. At the end of the period all of the females had open vaginas, which contained mucus and some cornified cells.

It is interesting to note that in all of these immatures the vaginas remained open much longer than was the case in the normal adults

(cf. table 1, chart 2). Whether this was due to the absence of progesterone in the juvenile animals remains to be seen.

TABLE 1. TIME (IN HOURS) REQUIRED FOR OPENING OF VAGINA AFTER INJECTION AND TIME (IN DAYS) DURING WHICH THE VAGINAS REMAINED OPEN AFTER LOCAL INJECTION OF WOMAN'S BLOOD. WEANLING GUINEA PIGS

No.	Weight material injected	Time of first opening (hours)	Length of time vagina remained open (days)
27	0.25 mgms. Stilbesterol	24	19
28	0.25 mgms. Stilbesterol	24	18
30	0.04 cc. Female Blood	36	17
31	0.06 cc. Female Blood	24	19
32	0.06 cc. Female Blood	24	16
33	0.06 cc. Female Blood	36	9
34	0.05 cc. Female Blood	48	20
35	0.08 cc. Female Blood	24	15
36	0.06 cc. Female Blood	36	11
37	0.06 cc. Female Blood	24	11
38	0.05 cc. Female Blood	36	13

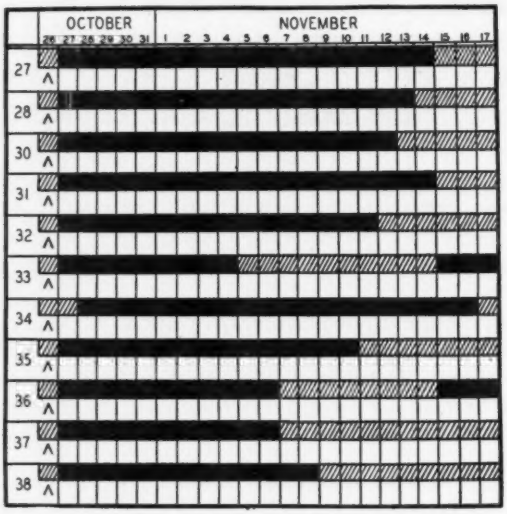

CHART 2. Showing time vaginas of 11 young guinea pigs remained open (solid areas) after injection (indicated by carets) of woman's blood. Data in table 1.

QUANTITATIVE EXPERIMENTS

In order that the method here described may be rendered most useful in the assay of blood estrogen it was necessary that some idea be gained as to the relation of known amounts of the hormone to the interval of time between the injection and the opening. The time of

re-closure is likewise a significant factor; but no accurate data were secured in the first series on this point. With extremely low dosages the delay in opening is greatly prolonged (96 hours with 4×10^{-7} mgm. of *estrogen* in 0.02 cc. of oil.) The opening may proceed only to the pin-point stage and close up again within a few hours.

For the injections a graded series of estradiol dipropionate in peanut oil was used. The concentrations were so calculated that the

TABLE 2. RELATION OF ESTROGEN DOSAGE TO TIME OF VAGINAL OPENING IN 14 WEANLING GUINEA PIGS (SERIES I) AVERAGING 192 GRAMS IN WEIGHT

Animal number	Weight	Dosage (mgms.) in 0.02 cc. oil	Time of opening (hours)
86	192.2	0.02	10.0
87	151.5	0.007	28.0
88	199.6	0.005	34.0
89	210.0	0.001	42.0
51	178.7	0.0002	48.0
56	200.0	0.0002	51.0
61	159.5	0.0002	50.0
52	198.7	0.0001	53.0
57	186.4	0.0001	54.0
58	209.0	0.00005	57.0
53	208.0	0.00002	58.0
59	203.4	0.00002	56.0
55	208.5	0.00001	60.0
60	180.0	0.00001	62.0
	192.0 Average wt.		

TABLE 3. SERIES II. RELATION OF ESTROGEN DOSAGE TO TIME OF VAGINAL OPENING IN 9 GUINEA PIGS AVERAGING 288.2 GRAMS IN WEIGHT

Animal number	Body wt. (grams)	Dosage (mgms.) in 0.02 cc. oil	Time of opening (hours)
67	245.4	0.002	35
71	252.4	0.002	38
65	358.6	0.0007	47
70	284.0	0.0007	41
64	314.1	0.0005	48
63	306.0	0.0002	50
69	264.6	0.0002	52
62	292.0	0.0001	52
68	260.3	0.0001	55
	288.2 Average wt.		

required dosages (from 2×10^{-3} to 2×10^{-7} mgm.) were each contained in 0.02 of oil.

Table 2 gives the data for the first series of 14 animals. In some cases a given dosage was administered to two or three different animals. It is seen that it required a somewhat longer time for the vagina to open with the smaller doses; but the difference in this respect between the animals receiving one gamma and those receiving one-thousandth of a gamma was surprisingly small.

A second series of 9 somewhat larger animals were similarly treated. The results, shown in table 3, closely parallel those of Series I (table 2).

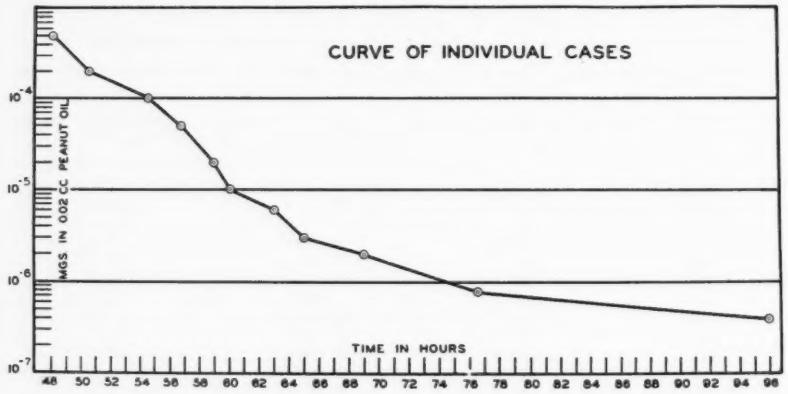

CHART 3. Curve of time of opening of vaginas of 11 weanling guinea pigs receiving varying doses of *estrogen*. Data in table 4.

TABLE 4. RELATION OF DOSAGE OF ESTROGEN TO TIME OF OPENING OF THE VAGINA IN 13 WEANLING GUINEA PIGS AVERAGING 192 GRAMS IN WEIGHT. EACH CIRCLE REPRESENTS A SINGLE CASE

Number	Weight (grams)	Dosage (mgms)	Time of opening (hours)
72	148.1	0.0005	48.0
73	152.1	0.0002	50.5
74	218.0	0.0001	54.5
75	205.0	0.00005	56.5
76	163.5	0.00002	59
77	197.2	0.00001	60
79	206.1	0.000006	63
80	196.0	0.000003	65
81	196.9	0.000002	69
82	151.5	0.0000008	76
83	175.7	0.0000008	77
84	199.6	0.0000004	96.1
85	210.0	0.0000002	did not open
	192.0 Av. wt.		
78	Control 192.2		Did not open.

Since the results thus far had shown that the threshold had not been reached by the lowest values mentioned (1×10^{-5}, mg. opening in 60 hours) a new series of 13 animals was treated with a new series of solutions graded from 0.5 gamma to 2 one-hundred-thousandth gamma (5×10^{-4} to 2×10^{-7} mg.). The results are presented in table 4 and plotted in chart 3. Chart 4 represents the composite curve of all 36 cases. Where an entry represents more than one animal (see tables) the average value is set down.

It is seen from an inspection of chart 4 that it is possible to grade the effects of varying dosages in terms of time interval between the hour of injection and the hour of opening of the vagina.

That 4×10^{-7} mgm. of estradiol diproprionate represents a value near the threshold for this compound is shown by the fact that with

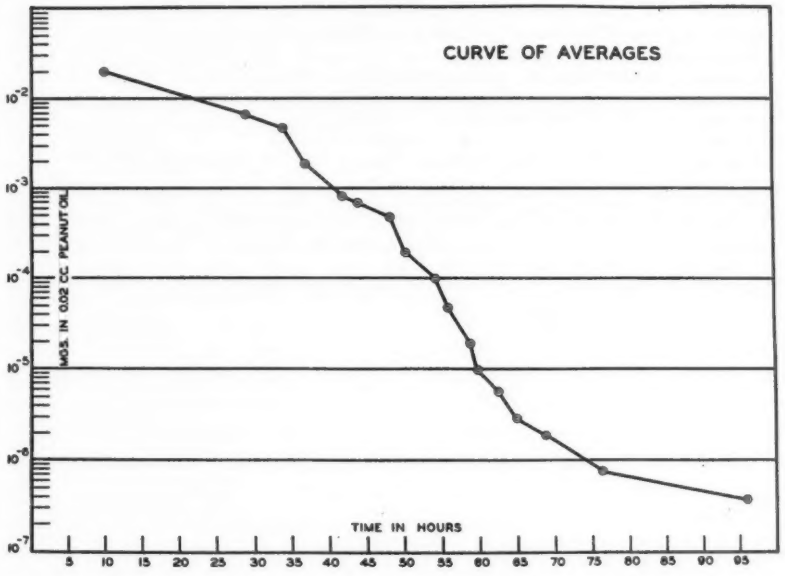

CHART 4. Curve based on all cases listed in tables 2, 3, and 4, showing relation of dosage of *estrogen* to time of vaginal opening in 36 weanling guinea pigs.

this amount only a pinpoint opening was effected (animal 84), while with half that dosage the vagina did not open at all (animal 85).

SUMMARY

A method of assaying estrogen by hypodermic injection of the vulvar region of weanling guinea pigs is here described. The technique is simple and the animals are not injured, hence need not be sacrificed. The endpoint consists of the disappearance of part or all of the vaginal closure membrane. The greater the dosage administered, the less time is required for the vagina to open and the more extensive is the involvement of the closure membrane. The threshold dose in these experiments was between 4×10^{-7} and 2×10^{-7} mgm. of the estrogen. The normal adult female is less well adapted to the test than weanlings about 200 grams in weight. Exploratory tests (not here detailed) indicate that the primed castrated adult female guinea pig is exceed-

ingly sensitive; and since it may be used over and over, may prove to be the animal of choice for these assays.

Indications are that progesterone in the normal cycle antagonizes estrogen, since, for example, in the preadolescent the vagina remains open for more than two weeks, only about 4 days in the normal adult.

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EXCRETION OF URINARY CORTICOIDS AND 17-KETOSTEROIDS IN THE NORMAL INDIVIDUAL[1]

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THE PRESENCE in human urine of substances which have the properties of adrenal cortical steroids has been demonstrated by many investigators. Perla and Marmorston-Gottesman (1931), Grollman and Firor (1932–33), Weil and Browne (1939a), Dorfman and Fish (1942), all have reported that extracts of urine of normal individuals are capable of either prolonging the survival period of adrenalectomized rats or of increasing their resistance to stress such as cold or histamine poisoning. In addition, it has been shown that these extracts will increase liver glycogen in the fasted adrenalectomized rat (Horwitt and Dorfman, 1943), prolong muscle performance (Shipley, Dorfman and Horwitt, 1943), and prevent water intoxication (Schiller and Dorfman, 1943).

Following damage, such as burns or surgical operation, Weil and Browne (1939b & 1940), using the Selye-Schenker cold exposure test, showed that the amount of these active substances is increased in the urine, and Venning, Hoffman, and Browne (1943 & 1944) demonstrated that extracts of urine obtained from patients after surgical operations also possess increased life-maintaining properties and the capacity to deposit glycogen in the liver of the fasted adrenalectomized rat.

Recently a method has been developed in this laboratory by Venning, Kazmin and Bell whereby small amounts of corticoids can be measured. It is a bioassay, based upon the ability of these hormones to cause deposition of glycogen in the livers of adrenalectomized mice. By means of this method it is now possible to follow the daily excretion of urinary glycogenic corticoids in the human being under normal and pathological conditions. A preliminary report of our findings has been made to the Associate Committee on Medical Research, National Research Council of Canada (1942–1946), and to the Conference on Metabolic Aspects of Convalescence including Bone and

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Wound Healing (1942–1946) held under the auspices of the Josiah Macy Jr. Foundation. The determination of the excretion of these compounds together with the 17-ketosteroids offers a means of studying certain aspects of adrenal function. This paper deals with the excretion of these adrenal metabolites in a group of normal individuals. The effect of sex and age and the daily variation in the output of these hormones have been determined.

METHOD

Two consecutive 24-hour specimens were collected without preservative and combined. An aliquot was removed for the determination of 17-ketosteroids. The remainder was acidified, extracted and assayed according to the method of Venning, Kazmin and Bell (1946). The activity is expressed in terms of glycogenic units excreted per 24-hours, one glycogenic unit being equivalent to the biological activity contained in 1 microgram of 17-hydroxy 11-dehydro corticosterone. Colorimetric measurements of the 17-ketosteroids were performed according to the method of Holtorff and Koch (1940), modified in that the time of color development was increased to 105 minutes and a correction factor for non-steroidal chromogens was applied.

RESULTS

Normal Women:

The excretion of glycogenic corticoids and 17-ketosteroids were followed in a group of 14 normal women aged from 21 to 45 years (Fig. 1). The urinary corticoid activity ranged from 25 to 65 glycogenic units per 24-hours with an average value of 41 units. The 17-ketosteroids ranged from 6–18 mg./24 hours with an average value of 11.6 mg. In two elderly women, aged 74 and 76 years, the urinary corticoids were 38 and 26 units respectively and the 17-ketosteroids 5.8 and 8.2 mg./24 hours.

Normal Men:

In 14 adult normal men between the ages of 20 to 51 years the excretion varied from 40 to 85 glycogenic units per 24 hours with an average value of 60 units (Fig. 1). The ketosteroids ranged from 10–25 mg./24 hours with an average value of 16.6 mg. In two older men, aged 77 and 81 years, in reasonably good health, the urinary corticoids were 37 and 20 units respectively, and the 17-ketosteroids 10.0 and 10.6 mg./24 hours.

Normal Male Children:

The urine from six newborn male infants was collected during the first four days of life and an amount of urine equivalent to a 12-hour specimen was injected into each mouse. At this level no activity could be detected in the specimen. In two male children between the ages of $2\frac{1}{2}$ and 3 years, there were already present in the urine corticoids equivalent to 35 to 40 glycogenic units. From the age of $5\frac{1}{2}$ years, adult male values were found. The ketosteroids were low in the

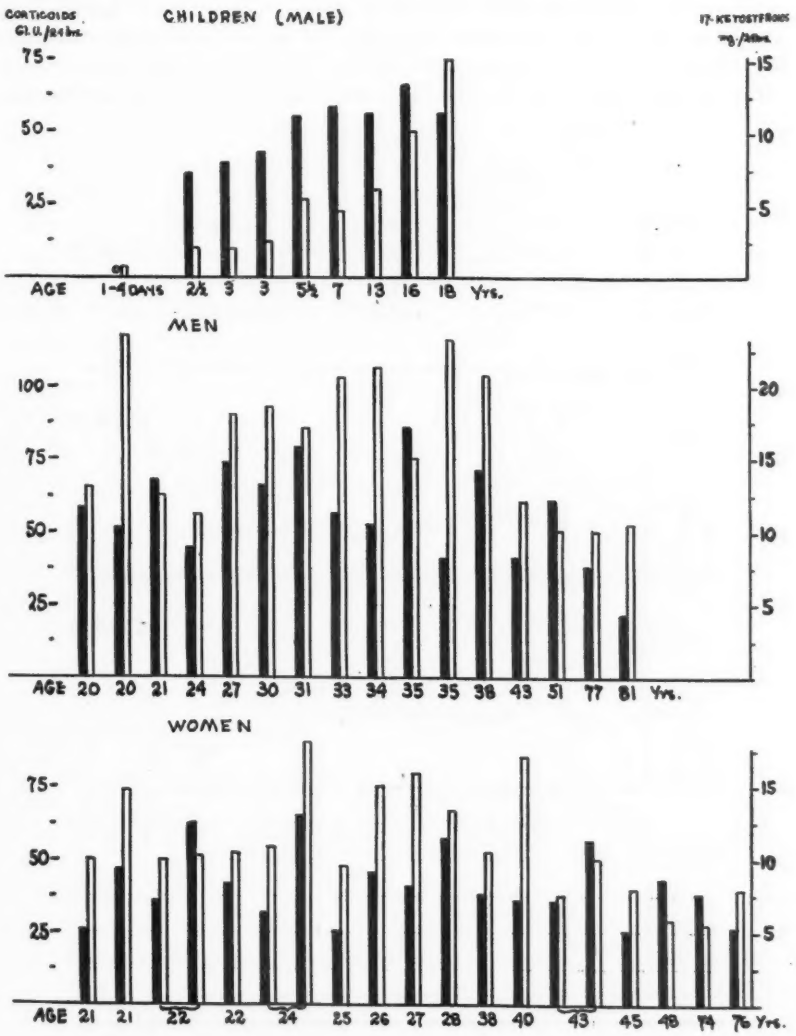

FIG. 1. Excretion of corticoids and 17-ketosteroids in a group of normal individuals. The black columns represent the daily excretion of corticoids in terms of glycogenic units, the white columns mg. of 17-ketosteroids.

younger children, increased in value with advancing age and reached adult values later than the corticoids.

Daily Variation in Excretion in the Same Individual:

The excretion of corticoids and 17-ketosteroids was followed over a period of two weeks in 4 normal individuals. Assays were done every

48 hours on pooled 24-hour specimens (Fig. 2). In case (1), a male age 30 years, the daily excretion of glycogenic substances varied from 35 to 86 units, with an average value of 61. The ketosteroids varied from 16 to 29 mg./24 hours. In case (2), a male age 43 years, the glycogenic

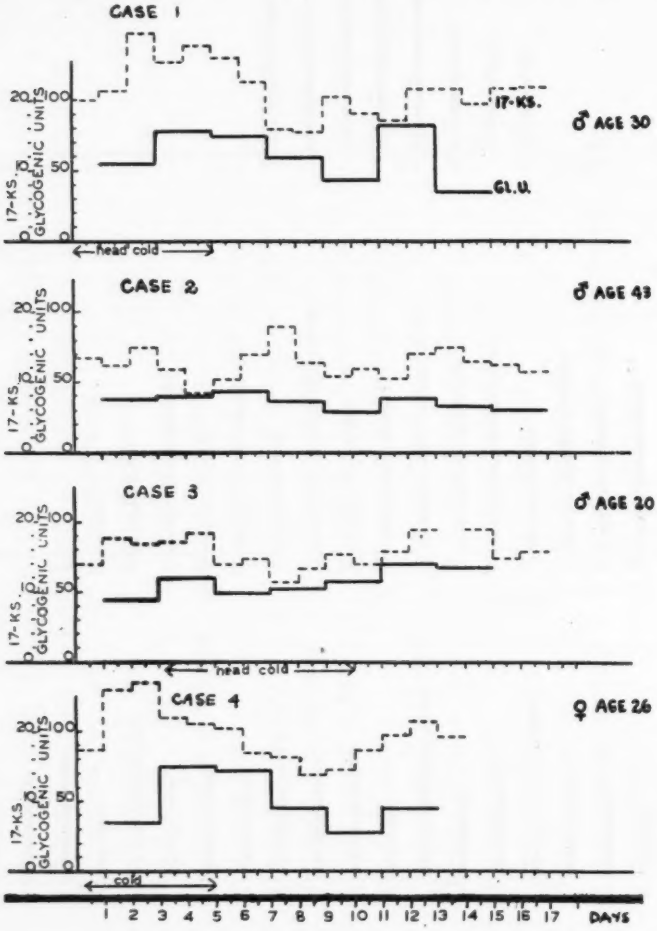

FIG. 2. Daily variation in excretion of corticoids and 17-ketosteroids in 4 normal individuals.

corticoids varied from 30 to 43 units with an average value of 36 units. The ketosteroids varied from 10 to 18 mg./24 hours. In case (3), a male age 20 years, the glycogenic substances varied from 46 to 71 units with an average value of 58 units. The ketosteroids ranged from 12 to 19 mg./24 hours. In case (4), a female age 26 years, the

glycogenic corticoids varied from 30 to 75 units with an average value of 50 units. The ketosteroids varied from 14 to 27 mg./24 hours. Cases (1), (3) and (4) developed head colds during the early part of the study. This, however, did not affect the level of excretion.

Variation During the Menstrual Cycle:

Two normal women were studied throughout the menstrual cycle (Fig. 3). In case A the urinary corticoids varied from 25 to 63 units with an average value of 47 units. The ketosteroids varied from 12.7 to 18.3 mg./24 hours. In case B the glycogenic corticoids varied from 20 to 55 units (average value 26 units) and the ketosteroids from 8.4 to 13.4 mg./24 hours. No changes were observed in the excretion of these substances in relation to the menstrual cycle.

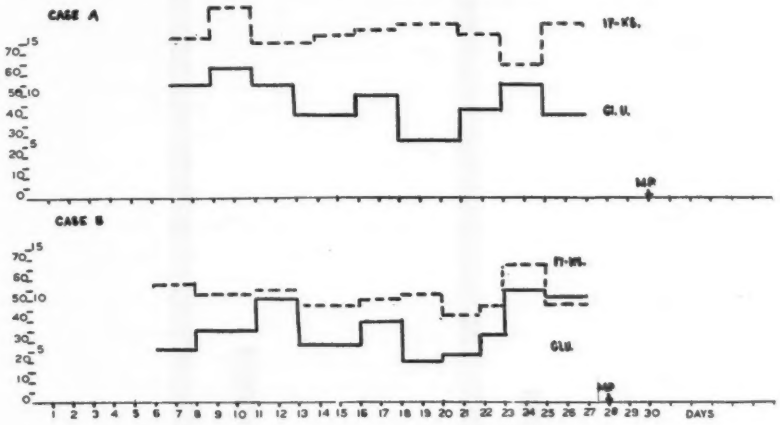

FIG. 3. Excretion of corticoids and 17-ketosteroids in the menstrual cycle in 2 normal women.

Effect of Exercise:

The effect of strenuous muscular exercise on the excretion of corticoids was determined (Fig. 4). Pooled urine was obtained from a group of 14 healthy army recruits before and after a 4 mile route march. The experiment was repeated the following week. The output of glycogenic corticoids was markedly increased following the strenuous exercise, from 46 to 160 glycogenic units per liter in the first experiment, and from 60 to 210 units in the second experiment. The slight increase in specific gravity of the urine did not account for the increased concentration of corticoids.

DISCUSSION

Both clinical and experimental evidence indicates that the adrenal cortex has various functions (salt and water metabolism, carbohy-

drate and protein metabolism, androgenic activity) which may be associated with the different types of steroids which it elaborates. As in the case of all studies of excretion products of hormones, there are numerous factors which affect the level of excretion other than the actual amount of active substances in the gland. In the case of the 17-

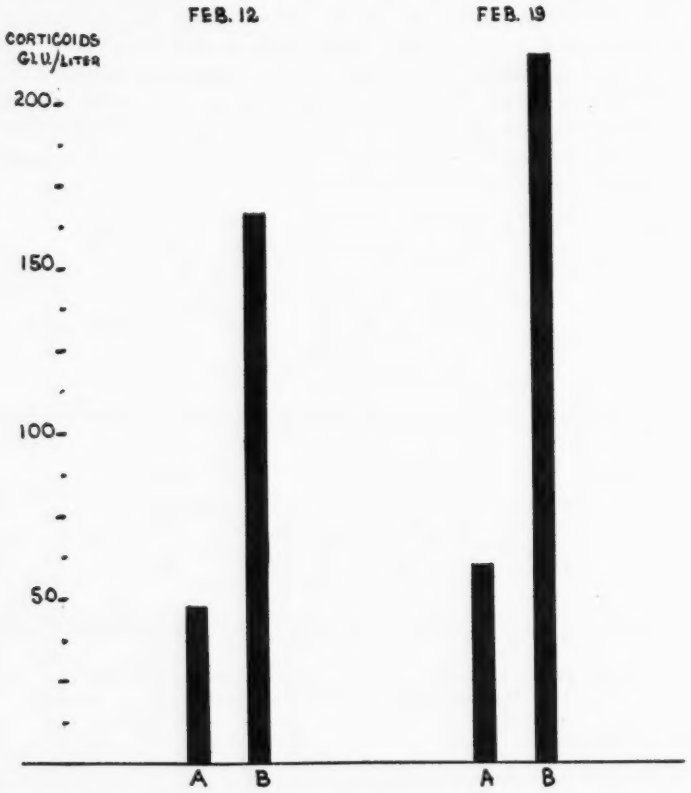

FIG. 4. Effects of strenuous exercise on the excretion of corticoids in a group of 14 Army recruits—A. before exercise, B. after exercise.

ketosteroids it is reasonably well established that they are derived from the testes and adrenal cortex in the male, and from the adrenal cortex in the female. While testosterone is considered by some to be the compound responsible for the urinary 17-ketosteroids of testicular origin, the adrenal precurosors of these metabolites have not yet been identified. The many steroids of both the androstane and the pregnane series which have been isolated from the urine of normal individuals are probably derived from the metabolism of these glands. What factors modify the pathway of metabolism of the active compounds, and

what effect variation in kidney and liver function have in determining the level of excretion are largely unknown.

With regard to the biological assay of corticoids, this method measures only those compounds which possess the property of affecting carbohydrate metabolism, i.e. causing deposition of glycogen in the livers of adrenalectomized animals. It will, therefore, not detect changes in adrenal function affecting compounds of the desoxycorticosterone type, or compounds having androgenic activity which do not possess this property. The chemical nature of these glycogenic corticoids excreted in the urine has not yet been determined. Venning, Hoffman and Browne (1944) isolated a fraction which had an activity in the cold exposure test approximately equal to that of corticosterone. This fraction contained a large part of the activity found in the original crude urinary extract. If the active compounds present in the urine are of an order of activity similar to that possessed by the steroids isolated from the adrenal cortex, then the actual amount of these substances excreted per day according to the present method is very small (25 to 85 micrograms of a compound possessing the activity of 17-hydroxy 11-dehydro corticosterone). This is of a different order of magnitude to the excretion of 17-ketosteroids which are excreted in milligrams per 24 hours. These active substances in the urine probably only represent a fraction of the amount elaborated in the gland itself. When adrenal cortical extracts are injected intravenously, approximately 10% of the activity can be recovered as measured by the cold exposure test. The next question is whether the activity in the urine parallels the changes in adrenal cortical activity. It has been demonstrated by Venning and Browne (1945) that in conditions such as Addison's disease and pan hypopituitarism the excretion of biologically active compounds of this type is so low as in many instances not to be detectable by this method; that the administration of adrenocorticotrophin causes a rise in excretion of these substances and that in Cushing's Syndrome, the values are greatly increased. The prompt rise in urinary corticoids after exercise and after stress of various types also suggests a parallelism between excretion and adrenal cortical function. The urine of the male infant in the first four days after birth has been shown to contain little or no activity in spite of the fact that in late pregnancy (Venning 1945) the mother's urine contains large amounts of this type of activity. The large size of the foetal adrenal and the morphological changes which occur in it after birth are well known. The present findings may indicate that the foetal adrenal does not form the types of adrenal hormones found later in life, and that there is also a change of function after birth. In this case the adrenal of the premature infant may be incapable of responding to stress on account of its inability to produce increased amounts of the type of compounds formed by the adult adrenal. This might lead to a relative deficiency. The dramatic response of a premature

twin moribund with bilateral bronchopneumonia reported by Stevenson (1942), and the reports of beneficial affects of cortical extract in premature infants, may thus find a physiological explanation in a qualitatively different type of activity of the foetal adrenal. How soon corticoid activity appears in the urine after birth is not yet determined. By the age of 2½ years the urine of male children contained appreciable amounts of glycogenic corticoids and at 5½ years adult levels were present. The 17-ketosteroids, on the other hand, rose slowly with increasing age following a curve similar to that observed by Nathanson et al (1941), and reached adult levels much later than the corticoids. From the studies of children and adults in this paper it is obvious that there is not necessarily a parallelism between the excretion of urinary corticoids and of 17-ketosteroids. Greater amounts of corticoids are excreted by males than by females and the difference is statistically significant. Some variation is seen in the excretion of these substances from day to day in the same individual, both in males and in females, but values remain within the normal range established by single assays in a number of different individuals. Similar daily variations are observed in the excretion of 17-ketosteroids. No correlation could be found with the rhythmic excretion of the sex hormones in the menstrual cycle. Only a few studies on older individuals were made but these indicate that there is a somewhat lowered output in old age.

Infection and trauma, such as burns or fractures, will increase the output of glycogenic corticoids and severe muscular exercise will also raise the level of these substances in the urine. As bed patients appear to have a more even output of these substances than do active normal subjects, it is possible that the adrenal cortex responds promptly to the stresses and strains of ordinary life, (exercise, emotions, etc.).

SUMMARY

Urinary 17-ketosteroids and glycogenic corticoids have been measured in a series of normal individuals. The assay method of Venning, Kazmin and Bell has been used for determining the urinary corticoids. It is based upon the ability of these hormones to cause deposition of glycogen in the livers of adrenalectomized mice, 1 glycogenic unit being equivalent to the biological activity contained in 1 microgram of 17 hydroxy 11-dehydro corticosterone. Normal adult males excrete between 40-85 units of glycogenic corticoids per 24 hours; normal adult females between 25-65 units per 24 hours.

No corticoids could be detected in the urine of male infants 1 to 4 days old, but amounts in the order of 35-42 units were found in the urine of of two male children between the ages of 2½ to 3 years. At 5½ years. the excretion of these substances had reached values found in adult males. In old age the excretion of corticoids is somewhat lower than in adult life. Some variation in the daily excretion of both corti-

coids and 17-ketosteroids was found in both sexes, however, no cyclic changes were observed in the menstrual cycle.

The excretion of corticoids and 17-ketosteroids vary independently.

ACKNOWLEDGEMENT

The authors are greatly indebted to Mrs. J. C. Bell, Miss E. Crotty and Miss M. P. Ripstein for their technical assistance.

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THE DISTURBANCE IN IODINE METABOLISM PRODUCED BY THIOCYANATE: THE MECHANISM OF ITS GOITROGENIC ACTION WITH RADIOACTIVE IODINE AS INDICATOR[1]

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FROM OBSERVATIONS made in this laboratory (Franklin, Chaikoff and Lerner, 1944) it was concluded that thiocyanate inhibits the iodine concentrating capacity of thyroid tissue *in vitro*. This conclusion was based on experiments in which surviving slices of thyroid tissue were suspended in a Ringer medium containing radioactive inorganic iodide (I^{131}) as a labeling agent.

More recently the goitrogenic action of thiocyanate *in vivo* has been investigated by Rawson, Tannheimer and Peacock (1944). These workers measured the uptake of radioactive iodine by rat thyroids that had been made hyperplastic by the administration of thiocyanate Average values of 56 and 87 per cent of the injected radioactivity were recovered in the thyroid gland of the control and thiocyanate-treated animals respectively.

In view of the apparent discrepancy between the *in vitro* and *in vivo* effects of thiocyanate, the action of this goitrogen on iodine metabolism has been reinvestigated.

I. *In Vitro* EXPERIMENTS

In the experiments of table 1, 250 or 300 mg. of sheep thyroid slices were suspended in 3 cc. of an oxygenated Ringer-bicarbonate solution containing radioactive inorganic iodide. No I^{127} was added to the medium, the only I^{127} present being that due to the unavoidable impurities in the reagents used in the preparation of the medium (approximately 0.05 micrograms).

In the control flask of experiment 1, 76 per cent of the radioactive iodine that had been added to the medium was taken up by the surviving thyroid slices in the course of 1 hour; 54 per cent was organically bound. In the presence of 10^{-3}M KSCN only 21 per cent of the

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TABLE 1. THE EFFECT OF THIOCYANATE ON THE *In Vitro* UPTAKE OF IODINE BY SURVIVING THYROID SLICES

(Sheep thyroid slices were incubated at 38° for one hour in a bicarbonate Ringer medium containing I^{131} as iodide. The slices were then separated from their medium and analysed.)

Expt.	Thyroid tissue in flask	KSCN content of Ringer solution	Per cent of I^{131} of Ringer's recovered in slices as:				
			Total organic	Thyroxine	Diiodotyrosine	Inorganic	Total
	mg.						
1	250	None	53.9			22.2	76.1
	250	10^{-3}M	8.7			12.2	20.9
2	300	None	39.3	5.8	33.5	9.9	49.2
	300	10^{-3}M	9.7	1.6	8.1	14.1	23.8
3	250	None	45.3			27.6	72.9
	250	10^{-3}M	7.2			9.5	16.7

Ringer's I^{131} was recovered in the slices at the end of 1 hour; the amount organically bound was reduced to 9 per cent. Similar results were obtained in experiments 2 and 3 (table 1). These observations confirm the results previously reported by Franklin, Chaikoff and Lerner (1944).

II. *In Vivo* EXPERIMENTS

The 16 rats recorded in table 2 were fed a diet containing 0.5 per cent KSCN for 18 and 24 days. During the 16-24 hours preceding the excision of their thyroids they received neither food nor KSCN. Approximately 2.5 microcuries of radioactive iodine (I^{131}) were injected

TABLE 2. THE EFFECT OF INGESTION OF KSCN WITH FOOD UPON THE UPTAKE OF INJECTED RADIOIODINE BY THE THYROID GLAND OF THE RAT

(All rats were fasted for 16–24 hours before being sacrificed)

Period KSCN fed	KSCN ingested (average)	Rat wt. when sacrificed	Thyroid weight	Time I^{131} in body	Per cent injected I^{131} recovered in thyroid gland as:				I^{127} found in whole thyroid gland as	
					Thyroxine		Total			
					Whole gland	per mg. gland	Whole gland	per gm. gland	Thyroxine	Total
days	mg.	gm.	mg.	hours					micrograms	micrograms
18	0 control	313	24	10.5	7.5	0.31	56.0	2.3	3.0	
		284	27		5.8	0.21	49.3	1.8	2.2	
		252	24		6.6	0.28	57.6	2.4	2.3	
		258	24		6.5	0.27	51.0	2.1	2.7	
	88	274	48	10.5	11.8	0.25	72.3	1.5	0.84	
	75	240	45		19.2	0.43	82.2	1.8	1.1	
	70	275	40		18.3	0.46	74.3	1.9	1.1	
	54	216	32		10.8	0.34	81.3	2.6	0.78	
24	0 control	300	29	4.5			35.2	1.2		12.4
		263	23				32.8	1.4		5.6
		330	24				24.8	1.0		9.3
		260	24				31.2	1.3		6.1
	56	259	50	4.5			79.5	1.6		2.7
	62	277	75				74.6	1.0		1.0
	70	281	54				84.5	1.5		2.3
	54	274	47				79.5	1.7		2.5

intraperitoneally 4.5 or 10.5 hours before the glands were removed. The samples of I^{131} used throughout this study contained no measurable amounts of I^{127}.

The feeding of KSCN for 18 and 24 days produced considerable enlargement of the thyroid gland. Although the uptake of I^{131} per whole gland was greater in the KSCN-treated rats than in their controls, this was not found to be the case when the comparisons were made on a per gram basis. There can be little doubt from the data presented in table 2 that under the particular conditions of this experiment the iodine-concentrating capacity per unit weight of thyroid tissue was not increased.

The failure of KSCN to depress the uptake of I^{131} by the intact thyroid gland is somewhat surprising in view of the *in vitro* effects of this drug described above. It should be noted that the rats received no KSCN during the 16 to 24 hours preceding the excision of the gland. This raised the question whether the rapid disappearance of KSCN from the tissues of the rat during the last 16 to 24 hours could account for the difference between the *in vitro* and *in vivo* results. Experiments designed to test this possibility are described below; the results are recorded in table 3.

With the exception of the rats of group A, which served as controls, all rats of table 3 received KSCN either intraperitoneally or per os. The rats of groups B and C were injected intraperiotoneally with 50 mg. of KSCN daily for 10 days. The rats of groups D and E ingested from 65 to 90 mg. of this drug per day for 10 days.

The rats of all 5 groups were deprived of food for the 24 hours previous to the removal of their thyroid glands. Each rat received intraperitoneally approximately 2.5 microcuries of radioactive iodine 5 hours before the gland was excised.

The rats of groups B and D differ from those of groups C and E in that the latter received an intraperitoneal injection of 50 mg. of KSCN 5 hours before their thyroid glands were excised.

Group B—As already stated, this group received KSCN by intraperitoneal injection for 10 days but none during the last day of the experiment i.e. the 24 hours preceding the excision of the gland. A comparison with group A shows a significant increase in the thyroid weight of the thiocyanate-treated animals. The average thyroid weight of the controls was 26.2 (range from 24 to 28.5 mg.) while the average for group B was 38.1 mg. (range from 32 to 45 mg.). Furthermore, the thyroids of the KSCN-treated rats showed the same increase in the uptake of I^{131} above their controls that was exhibited by the 18 and 24-day groups recorded in table 2. Recoveries of the injected radioactive iodine by the thyroid glands of the rats of group B ranged from 52 to 78 per cent (average 63); in the controls (group A) recoveries ranged from 31 to 66 per cent (average 48). However, when the uptake of I^{131} by the thyroid glands of controls and KSCN-treated animals

was compared on a per gram basis, no significant difference was observed. The average recovery per millgram was 1.83 per cent for group A and 1.69 per cent for group B.

Group C—In addition to intraperitoneal injection of KSCN for 10 days, the rats of this group received an intraperitoneal injection of

TABLE 3. ALL RATS EXCEPT THOSE OF GROUP A RECEIVED KSCN EITHER INTRAPERITONEALLY OR ORALLY FOR TEN DAYS. THEY WERE FASTED FOR THE NEXT 24 HOURS AND IMMEDIATELY THEREAFTER SACRIFICED. THEIR TREATMENT ON THE 11TH OR LAST DAY OF SURVIVAL IS DESCRIBED IN THE FIFTH COLUMN BELOW.

1	2	3	4	5	6	7	8	9	10	11
		KSCN administration			Weight of animal				Per cent recovery of injected I^{131}†	
Group	Rat no.	Daily for 10 days		Amount injected intraperitoneally on last day 5 hours before death of animal	At start	At end	Thyroid weight*	Plasma SCN†	In whole thyroid	Per gm. of thyroid
		Route	Amount							
			mg.	mg.	gms.	gms.	mg.	mg. per cent		
	1				308	305	26.5		31	1.2
	1				260	264	25.5	2.2§	47	1.8
A	3		None	None	252	265	28.5		66	2.3
	4			(Controls)	279	291	28.5	2.4	57	2.0
	5				252	270	24		34	1.4
	6				270	248	24	2.5	53	2.2
	7				255	251	39		68	1.7
	8				280	275	45	3.2	65	1.4
B	9	Intraperito-			285	286	36		65	1.8
	10	neally	50	None	250	247	44	2.1	78	1.8
	11				260	246	32		57	1.8
	12				258	250	33	2.3	52	1.6
	13				251	260	33		0.7	0.021
	14				308	285	33.5	17.8	1.8	0.054
C	15	Intraperito-	50	50	262	257	33		2.0	0.061
	16	neally			266	251	27	18.6	0.3	0.011
	17				268	273	30		0.6	0.020
	18				260	251	29.5	20.8	1.5	0.051
	19		79		286	288	44		76	1.7
	20		83		267	250	39	2.1	66	1.7
D	21	Orally‡	83	None	263	248	39		69	1.8
	22		65		268	212	28.5	2.1	51	1.8
	23		87		258	262	45	2.8	50	1.1
	24		85		280	284	49		2.1	0.043
	25		72		256	250	35	21.1	0.8	0.023
E	26	Orally‡	69	50	258	232	35		0.8	0.023
	27		81		266	266	54	21.4	1.5	0.028
	28		90		270	260	37		1.2	0.032
	29		76		272	282	54	20.8	1.6	0.030

* Weighed to the nearest half milligram.
† The radioactive iodine was injected 5 hours before the rats were sacrificed. At the time they were sacrificed they had been fasted for 24 hours.
‡ The KSCN was mixed with the diet in the proportion of 5 mg. per gram of diet.
§ Plasma samples from two animals were pooled.

50 mg. of KSCN 5 hours before they were sacrificed. Although the increase in weight of the thyroid was slight, if any, a 42 fold decrease in the amount of the injected I^{131} taken up by the glands was observed.

Group D—The results here are similar to those of group B. The only difference between groups B and D was the route of KSCN administration; neither received the drug on the last day. As in group B, the glands of this group showed an average weight increase of 50 per cent. The whole gland of the rats of this group had a greater than normal

(control) avidity for radioactive iodine. No difference between groups D and A was observed, however, when the uptake of I^{131} was compared on a per gram basis.

Group E—Enlargement of the glands was quite prominent in this group. These rats received KSCN 5 hours before removal of the gland as well as during the 10 preceding days. The uptake of I^{131} by thyroid tissue was depressed to a degree equal to that of group C. The average recovery of radioiodine from the glands of group E was 1.3 per cent as compared with 48 per cent from the control glands (group A).

The significance of this last injection of KSCN just 5 hours before

TABLE 4. THE EFFECT OF A SINGLE INJECTION OF 50 MG. OF KSCN UPON THE UPTAKE OF I^{131} BY THE THYROID GLAND[1]

Rat	KSCN[1]	Per cent recovery of I^{131} in:	
		Whole gland	Per mg. of thyroid tissue ×100
1	None	28	88
2	None	28	132
3	None	30	118
4	None	12	48
5	None	42	152
6	50 mg.	0.13	0.72
7	50 mg.	0.28	1.85
8	50 mg.	0.21	1.14
9	50 mg.	0.18	1.03
10	50 mg.	0.21	1.20

[1] Both the Radioiodine and the 50 mg. of KSCN were injected intraperitoneally 5 hours before the animals were sacrificed.

examination of the gland is clearly brought out by comparing the uptake of radioactive iodine by the glands of groups D and E. The glands of group D contained from 50-76 per cent of the injected I^{131}; those of group E from 0.8 to 2.1 per cent.

Relation of Plasma Thiocyanate Level to Uptake of Radioiodine—An examination of the concentration of thiocyanate in plasma (table 3) offers confirmation of the view that the increased recovery of I^{131} by the goitrous whole glands of fasted rats (table 2; groups B and D of table 3) can be explained by the rapid elimination of KSCN from the circulation. An increased uptake of I^{131} by the thyroid glands was observed only when the concentration of plasma thiocyanate was near normal. When, however, the uptake of radioactive iodine was depressed, plasma levels of thiocyanate ranged from 17.8 to 21.4 mg. per cent as compared to control values of 2.2-2.5 mg. per cent.

The most striking example of thiocyanate inhibition upon the uptake of iodine *in vivo* is presented in table 4. The results show the effect of a single administration of KSCN five hours before the animals were sacrificed. These rats received no previous KSCN-treatment. The drug in this case reduced the percentage recovery of I^{131} in the

glands from an average of 28 per cent (controls) to 0.2 per cent in the injected animals. This represents a 140 fold decrease in the uptake of radioiodine.

The Effect of Prolonged KSCN Feeding on Thyroxine Content of the Thyroid Glamd—The rats of group B (table 5) were fed a diet containing 0.5 per cent KSCN for 18 days whereas those of group A received the identical diet minus the KSCN. All animals were fasted for

TABLE 5. THE EFFECT OF THIOCYANATE FEEDING FOR 18 DAYS ON THE IODINE CONTENT OF THYROID GLANDS
(The rats were fasted for 16–24 hours before they were sacrificed)

Group	KSCN in diet*	Rat weight	Thyroid weight	Thyroxine iodine		Total iodine	
				Whole gland	mg. per cent†	Whole gland	mg. per cent†
		gm.	mg.	micro-grams	mg.	micro-grams	mg.
A	None (control)	232	23	1.6	7.0	6.7	29
		220	29	1.6	5.5	5.9	21
		224	27	1.3	4.8	5.7	21
		248	32	2.3	7.2	8.6	27
		211	18	1.7	9.4	7.2	40
		240	27	2.4	8.9	9.3	35
B	0.5 per cent	223	55	0.82	1.5	3.1	5.7
		221	64	0.75	1.2	2.8	4.4
		228	78	0.70	0.9	3.0	3.8
		213	48	0.60	1.3	2.4	5.0
		212	64	0.43	0.7	1.8	2.8
		229	69	0.42	0.6	2.2	3.2

* The diets were identical in both groups except for the addition of 0.5 per cent KSCN.

† mg. per cent refers to mg. per 100 gm. wet thyroid tissue.

16-24 hours before they were sacrificed. The data recorded in table 5 show that prolonged feeding of KSCN depresses the thyroxine and total iodine content of the glands of these rats. The average of the values for thyroxine iodine in the thyroids of control rats was 1.8 micrograms or 7.1 mg. per 100 gm. of wet tissue. In the KSCN-treated rats the corresponding values were 0.62 micrograms and 1.0 mg. per cent. Likewise the total iodine content was reduced in the thyroids of rats fed KSCN.

Effect of KSCN Feeding on Protein-bound Iodine of Plasma—Table 6 shows that the feeding of a diet containing 0.5 per cent KSCN depresses the concentration of protein-bound iodine in plasma. This was particularly marked in the animals that received the KSCN for 21 days.

DISCUSSION

The results of the present investigation confirm the conclusion drawn earlier, namely that in the presence of KSCN the uptake of

TABLE 6. THE EFFECT OF THIOCYANATE ON PROTEIN-BOUND PLASMA IODINE OF THE RAT

(The rats were fasted for 24 hours before they were sacrificed)

Experiment	Rat	KSCN		Rat weight	Thyroid weight	Protein-bound iodine of plasma
		Percentage in diet	Duration of feeding	gm.	mg.	micrograms per cent
1	1	None	21 days	240	16.5	2.9
	2	None	21 days	272	18	3.2
	3	None	21 days	240	21	3.2
	4	None	21 days	260	21	3.6
	5	None	21 days	257	23	3.3
	6	0.5	21 days	252	41	1.0
	7	0.5	21 days	251	31	1.8
	8	0.5	21 days	232	33.5	1.7
	9	0.5	21 days	252	32	1.6
	10	0.5	21 days	242	29	1.5
2	11	None	14 days	236	19.5	4.0
	12	None	14 days	286	23	3.7
	13	None	14 days	266	15	2.9
	14	None	14 days	276	23	2.8
	15	None	14 days	276	21	3.6
	16	None	14 days	280	22	3.0
	17	0.5	14 days	236	47	2.2
	18	0.5	14 days	252	42	2.1
	19	0.5	14 days	266	43.5	2.0
	20	0.5	14 days	254	41	2.0
	21	0.5	14 days	266	51	2.5
	22	0.5	14 days	246	53.5	2.0

radioactive iodine by thyroid tissue is depressed (Franklin, Chaikoff and Lerner, 1944). This was found to be the case irrespective of whether the action of KCSN was tested *in vivo* or *in vitro*. In the latter, KSCN is added directly to the medium in which the thyroid slices are bathed and in such experiments there can be no question that KSCN is present in relatively high concentrations at the time the radio active iodine is penetrating the thyroid slice. In the case of the intact animal it is shown here that thiocyanate is rapidly removed from the the circulation, but so long as a high concentration of thiocyanate is present in plasma the uptake of radioactive iodine by the thyroid gland is definitely depressed. So pronounced is the inhibitory action of this drug that it is observed even after a single injection, provided that the radioactive iodine be administered at a time when excess amounts of thiocyanate are still present in the circulation.

Although an increased uptake of radioactive iodine by whole thyroid glands of KSCN-treated rats was also found in the present investigation (this finding is in agreement with that of Rawson, Tannheimer and Peacock, 1944) this effect was observed only when the in-

terval after the last injection of thiocyanate was prolonged to the point at which its disappearance from the circulation had occurred. Even under these conditions, however, the increased uptake was not apparent when the gland's content of I^{131} was expressed on the basis of unit weight of tissue.

Thus, as judged by studies with the radioactive isotope of iodine, KSCN, when fed to rats maintained on a diet low in iodine, interferes with the capacity of the thyroid gland to remove iodine from the circulation. As shown by the *in vitro* experiments, KSCN also interferes with the conversion of inorganic iodine to diiodotyrosine and thyroxine. As was to be expected from these findings with the radioactive isotope of iodine the thyroxine (I^{127}) content of the thyroid gland and the level of protein-bound iodine of plasma were reduced in rats fed KSCN for several weeks.

EXPERIMENTAL

In Vitro Experiments—The preparation of the Ringer-bicarbonate medium and the method of slicing and incubating thyroid tissue have been described previously (Morton and Chaikoff, 1944). The slices were separated from their medium at the end of the run by pouring the entire contents of the reaction vessel through a coarse filter. The slices were then picked up with forceps, dipped several times in a non-radioactive Ringer's solution to remove adhering I^{131} and touched momentarily to a piece of filter paper to remove excess liquid.

In experiments 1 and 3 (table 1) in which the I^{131} was fractionated into organic and inorganic iodine, the slices were transferred to a 25 cc. Erlenmeyer flask and the hydrolysis and separation carried out as previously described (Schachner, Franklin and Chaikoff,1944). In experiment 2 (table 1) the separation of I^{131} into thyroxine, diiodotyrosine and inorganic iodine was carried out by the procedure of Perlman, Morton and Chaikoff (1941).

Composition of Diets—The diet fed to the rats had the following composition: 67.5 per cent whole wheat, 15 per cent casein, 10 per cent whole milk powder, 1 per cent NaCl, 1.5 per cent chalk, 5 per cent hydrogenated vegetable oil and 50 cc. fish oil per 8 kg. of diet. The iodine content of this diet was approximately 0.1 microgram per gm.

The thiocyanate diet was prepared by mixing in a ball mill 100 gm. of the above diet with 0.5 gm. of KSCN.

Protein-bound Iodine of Plasma—The proteins of 3 cc. of plasma were precipitated with zinc hydroxide and the iodine determined as previously described (Taurog and Chaikoff, 1946).

Thyroid Iodine—The separation of thyroid iodine into thyroxine and non-thyroxine fractions has also been described elsewhere (Taurog and Chaikoff, 1946).

Thiocyanate Level of Plasma—A modification of the Schreiber method (1925) was used. Nine cc. of a 5 per cent trichloracetic acid (TCA) solution were added to one cc. of plasma and the mixture shaken and centrifuged. The precipitate was washed once with 5 cc. of a 4.5 per cent TCA solution and the supernatants combined and made to volume with 4.5 per cent TCA. Five cc.

of this supernatant liquid were now mixed with one cc. of ferric nitrate reagent (5 gm. crystalline ferric nitrate and 2.5 cc. of concentrated nitric acid per 100 cc. of solution) and the color read in a Klett-Summerson colorimeter (filter 42) within 5 minutes. These readings were compared with known KSCN samples that had been standardized against silver nitrate.

SUMMARY

The *In Vitro* accumulation of inorganic iodine by surviving thyroid tissue, as measured with radioactive iodine, is inhibited in the presence of 10^{-3}M KSCN. Under these conditions the drug also depresses the synthesis of diiodotyrosine and thyroxine.

The iodine-concentrating capacity of the intact thyroid gland, as measured by the uptake of radioactive iodine, is depressed in rats maintained on a low iodine intake by KSCN administered either per os or by injection provided high concentrations of the drug are still present in the circulation. So pronounced is this action that it was observed at an early interval after a single injection of KSCN.

Administered KSCN is rapidly removed from plasma, its elimination being practically complete in 24 hours.

Sixteen to twenty-four hours after the last KSCN administration, at a time when an increased concentration of KSCN can no longer be demonstrated in plasma, the enlarged whole gland of the thiocyanate-treated rat has a greater than normal capacity for concentrating administered radioactive iodine. This increase is not apparent, however, when the uptake of I^{131} is calculated on a per mg. basis.

The thyroxine content of the thyroid gland and the concentration of protein-bound iodine of plasma are reduced in rats chronically treated with KSCN.

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THE INFLUENCE OF INGESTED ESTROGENS ON FEED INTAKE, METABOLIC RATE AND LIPEMIA IN MALE FOWL

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DURING a series of experiments which were designed for the purpose of studying the several reactions of male birds to treatments with synthetic estrogens, it was repeatedly observed that the experimental birds had a notably greater feed intake than their controls. Similar observations were made by Thayer, Jaap and Penquite (1944-1945) but left by these authors as an unexplained fact, whereas Kempster and Turner (1945) argued that augmented feed intake would be a reasonable corollary to higher fat metabolism per se. Since the control birds of the present experiments weighed on the average as much as the treated birds, gained approximately as much in weight due, undoubtedly, to the nearly equal fat deposition and metabolised approximately equal amounts of fat this explanation seemed inadequate and a study of this phenomenon was therefore undertaken.

MATERIAL AND PROCEDURE

A group of 140 Barred Plymouth Rock males which were 31 weeks old at the beginning of the test were, on the basis of equal body weight, divided into seven pens of 20 birds each and randomized into battery cages. The basal ration which has been used throughout these experiments was composed as follows:

Commercial rearing mash	50
Ground Wheat	30
Ground Oats	10
Ground Barley	10
Peanut Oil	1

As all tests were of short duration it was assumed that the vitamin content of the rearing mash made the whole ration adequate in this respect. The peanut oil served in the experimental rations as solvent for the estrogens which in this experiment were dimethyl ether of diethylstilbestrol and a 2:1 mixture of dienestrol diacetate and dianisyl hexadiene which hereinafter will be referred to as DED and DA respectively. The dissolved estrogens were thoroughly mixed into the ration in a mechanical mixer and its chemical analysis was as follows:

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Water	9.6%
Ash	3.9%
Protein	14.6%
Fibre	4.7%
Carbohydrates	63.1%
Fat	4.1%

The scheme of the experiment provided for three levels of the two estrogens, namely 13.22 mg., 11.01 mg., and 8.81 mg. per 100 gm. of feed, to be fed to corresponding groups of birds and a control group which received no estrogen. The experiment lasted 12 days.

EXPERIMENTAL

The weight of each bird was secured at the beginning and termination of the test as well as the weight of feed intake and from these

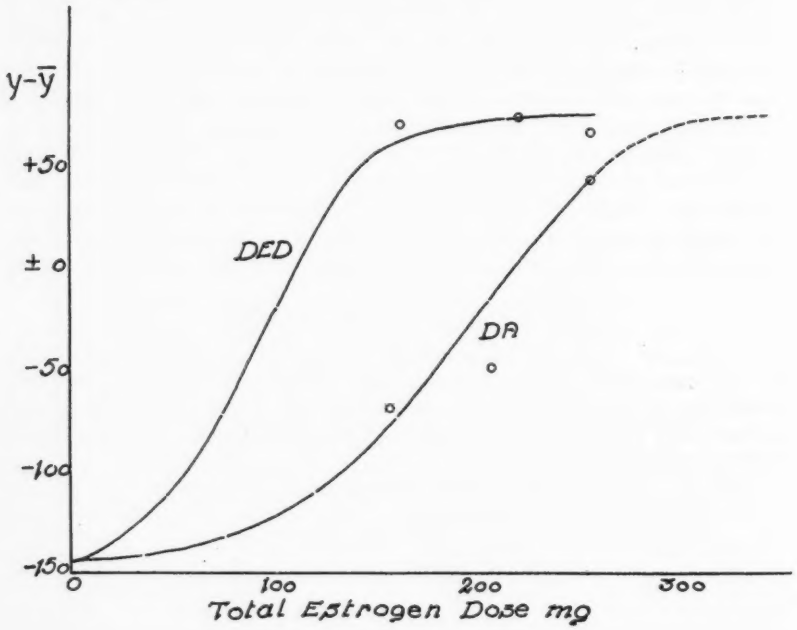

FIG. 1. Effect of Estrogens on Feed Intake.

data the regression of mean body weight (χ_1) and weight of gain (χ_2) on weight of feed intake (y) was calculated which gave the following formula for expected feed intake

$$\bar{y} = 412.9 + .3878\chi_1 + 1.7875\chi_2$$

Difference between weight of observed and expected feed intake was determined for each bird as $y - \bar{y}$.

The mean results of these determinations for each group are plotted in Fig. 1 with tentative curves for the effect of the two estrogens and the average performances of the seven groups are shown in table 1.

A variance analysis of the individual $y - \bar{y}$ values disclosed a highly significant statistical difference in feed intake not only between treated birds and their controls but between the effectiveness in this respect of the two estrogens and between dosages as well. It will be seen from Fig. 1 that on a total intake of 164.8 mg. the DED curve is probably approaching its asymptote whereas a correspondingly effective dose of DA would be approximately 300 mg.

In order to understand the nature of the rise in feed intake consequent to estrogen treatment, the metabolic rate of birds, as measured

TABLE 1. AVERAGE PERFORMANCE OF SIX GROUPS OF ESTROGEN TREATED MALE BIRDS AND THEIR CONTROL GROUP OVER 14 DAYS

Estrogens	DED			DA			Control
Group	1	3	5	2	4	6	7
Mean Body wt. gm.	3336	3323	3322	3348	3308	3309	3261
Gain gm.	101.4	106.8	56.1	104.5	145.3	90.0	88.4
Abd. fat gm.	82.3	84.3	70.5	87.3			63.6
$y - \bar{y}$ gm.[1]	+66.0	+72.8	+70.2	+42.2	−46.3	−69.8	−141.1
Total estrogens mg.	257.1	221.8	164.8	256.5	206.8	157.5	

[1] See text for explanation of the significance of these values.

by cardiac rate was studied prior to and after such treatment. Boas and Landauer (1933) employed the heart rate technique in assaying basal metabolism in normal and frizzle fowl. Winchester (1940) found a rough parallelism between fasting heat production and heart rate and the failure of Mathews et al (1943) to demonstrate any effect whatever on blood pressure and heart rate after oral administration of diethylstilbestrol may have been due to ready elimination of this estrogen from the intestinal tract. Lorenz (1945) Jaap and Thayer (1944) and Bird (unpublished data) found this estrogen, when fed, of practically no value for fattening, growth and feminizing effect in mature birds, whereas Munro and Kosin (1940) and Jaap and Thayer (1944) demonstrated its effectiveness on oviduct growth in baby chicks.

The cardiac rate was studied on nine two-year old B.P.R. males. These were placed in battery cages and fed the above mentioned basal ration. After four days their cardiac rate was determined on a "Victor" electrocardiograph. The birds were placed on their backs in a cradle, the electrodes strapped to the proximal surface of the ulnar radial musculature (Lead 1) and their heads covered with a dark cloth. All birds were allowed fully 15 minutes of complete rest prior

to determinations. Four of the birds had been fed two doses each of crystalline DED, 40 mg. per dose, in capsules on January 10th and 14th, the other five at that time serving as controls. The heart rates were determined on March 2nd and 4th and showed a mean rate of 159 b.p.m. for those that had been estrogen treated in January and a mean rate of 236 b.p.m. for their controls. On March 5th DED was added to the ration of all birds at the rate of 14.32 mg. per 100 gm. feed, and electrocardiograms secured on March 8th, 14th and 20th. No further lowering of heart rate occurred in the birds that were estrogen treated in January whereas the rate gradually declined in their controls from 236 b.p.m. to 170 b.p.m. over the fourteen days

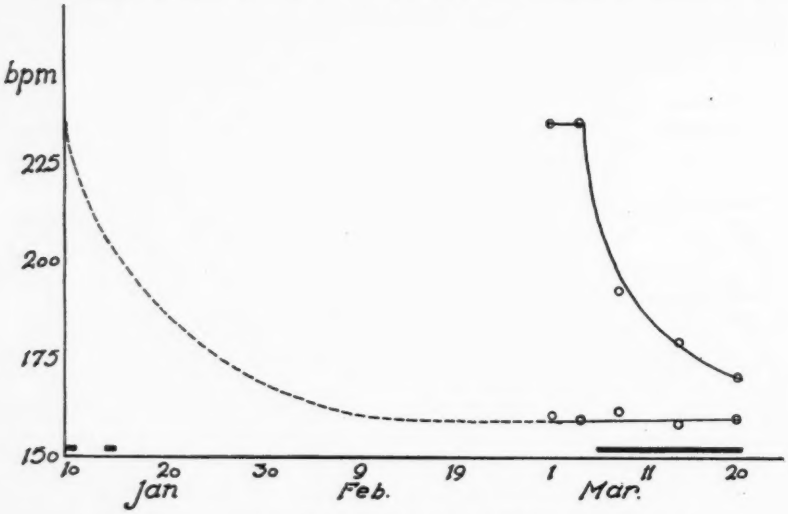

FIG. 2. Cardiac Rate Previous to and after Estrogen Treatment.

period during which time the mean intake of estrogen was 295.1 mg. These events are graphically presented in Fig. 2, which shows that estrogen reduces basal metabolic rate, as measured by heart rate, about 33 per cent within a relatively short span of time and that this effect is maintained for more than eight weeks. This chronic effect is undoubtedly due to mobilization of the physiologically large amounts of material which Pugsley and Bird (unpublished data) have found to be present in the depot fats of similarly treated birds.

It has repeatedly been shown by Warner and Edmond (1917) Lorenz et al (1938 a, b) Zondek and Marx (1939) Landauer et al (1939) Entenman et al (1940) Flock and Bollman (1942) and Bird (unpublished data) that one of the immediate effects of estrogen treatment is the appearance of severe lipemia. Zondek and Marx (1939) concluded that such lipemia is independent of nutrition since it occurs in starved as well as in fed birds whereas Lorenz et al (1938 a)

found that the level of fat in the diet had some effect on neutral fat and free cholesterol in the blood. The fat in the faeces of the nine males was determined both prior to and after the estrogen treatment in March, the mean values of which were as follows:

	Per Cent Fat in Faeces	
	Prior to estrogen in March	After estrogen in March
Estrogen in January	2.41	2.21
Control in January	2.65	2.67

There were no statistical differences between these determinations and although they were based on very limited numbers of observations it still seems warranted to conclude that the estrogen treatment at least did not induce a more intense extraction of fat from the contents of the intestinal tract and that fat in the ration, therefore, did not furnish the material for the estrogen induced lipemia.

DISCUSSION

In male birds which have been treated with estrogens three of the ensuing events are (a) a severe depression of metabolic rate (b) a lipemia as pronounced as that which occurs in females at puberty and (c) a markedly greater feed intake. Selye et al (1935) and Goldsmith et al (1945) and others found enlargement of the adrenal cortex after estrogen therapy. Bauman and Marine (1945) showed that the adrenal cortex exercises an inhibitory control over the thyroid and Lang et al (1940) as well as Lewis et al (1940) demonstrated that adrenal cortical hormone therapy raised blood glucose level and lowered the respiratory quotient in adrenalectomized rats, dogs, and in patients with Addison's disease. The very pronounced retardation of heart rate in fowls after estrogen treatment is probably, therefore, an indirect effect via the adrenal cortex and the depressed metabolic rate will in its turn make carbohydrates available for synthesis of fat. Since estrogen treatment of males induces in them the physiology of pubescent females of which severe lipemia is the most characteristic event and since such blood fat cannot be utilized by males in the formation of yolks its only other outlet is to the adipose tissues. The estrogens were in these experiments fed in physiologically excessive doses and the whole train of events was, therefore, maintained at an accelerated tempo leading to an acute and persistent demand for carbohydrates while the estrogen therapy lasted and consequently a desire for elevated feed intake which characteristically is proportionate to the potencies of the various estrogens and to their dose levels. The lipemia, which Zondek and Marx found in starved males should probably be regarded as an emergency utilization of depot fats in the total absence of nutritional carbohydrates.

SUMMARY

Feeding of relatively small doses of dimethyl ether of diethylstilbestrol produced a 33 per cent chronic depression of basal metabolism

as indicated by lowered cardiac rate. Massive doses of 21 mg. per day of the same estrogen continued for 14 consecutive days achieved similar acute results. This depression of metabolic rate was paralleled by enlarged feed intake which was in proportion to the potency of the estrogen employed and to dose level. In these experiments it was found that dimethyl ether of diethylstilbestrol had about twice the potency of a 2:1 mixture of dienestrol diacetate and dianisyl hexadiene as measured by level of feed intake.

The limited evidence available suggests that feeding of dimethyl ether of diethylstilbestrol did not affect fat extraction from the contents of the intestinal tract.

The probability that the female type of lipemia which occurs in estrogen treated males is sustained by synthesis of fat from nutritional carbohydrates which are made available for this purpose by lowered metabolic rate and elevated feed intake is briefly discussed.

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NOTES AND COMMENTS

ESTROGENS AND THE SEX SKIN IN THE MALE CHIMPANZEE

In the past decade the wide fluctuations in size and color of the sex skin of the females of various species of primates have been the subject of many investigations. It has become well established that tumescence in the female chimpanzee (Pan satyrus) and in the female chacma baboon (Papio porcarius) is not only coincident with the follicular phase, but may also be artificially produced with various estrogens. Gilbert (1944) has recently shown that the sex skin of the male chacma baboon is also responsive to estrogens. She found that in the intact adult male the subcutaneous injection of 1.0 mg. per day (see Table I) of estradiol benzoate in sesame oil produced a definite swelling of the perianal skin and reddening of the penis in one animal and slight swelling in the other two animals used. Increasing the dose led to definite but not measureable swelling and to edema of the penis in all animals. After castration the threshold rapidly diminished and two months after the operation, one tenth the minimum dose used in the intact animals was sufficient to cause a measureable amount of swelling in all of five castrated animals and in four of these there was also edema of the penis. Her results are summarized in Table I where it will be seen that the dose of estrogen given the castrate males was 2.5 times the dose necessary to produce maximal tumescence in the castrate female, while the doses used in the intact males were 25 and 125 times the optimal amount for replacement therapy in the castrate female (Gillman, 1942).

In a recent study of some of the effects of estrogenic therapy in the castrate male chimpanzee (Clark and Birch, 1945; Clark, 1945) no indication of perianal swelling, of penile edema, or of penile reddening was found. Since our negative results might have been due to too low a dosage, we decided to repeat the study using much larger amounts.* Two chimpanzees were used: Don castrated at the age of 22 months, nine and one-half years old when first treated with alphaestradiol and eleven and one-half years old at the conclusion of the experiment; Shorty, whose age was estimated at about 12 years at the end of the experiment. Both are thus young adults (Yerkes, 1943). Since it was necessary to use a drug effective orally, ethinyl estradiol was employed. The various doses used are listed in Table I. It will be seen that the smallest dose of ethinyl estradiol was twice the amount necessary to produce maximal tumescence of the sex skin of the castrate female chimpanzee and the largest was 180 times the replacement dose. At no time did we find any change in the sex skin or penis. The size of the flaccid penis and of the erect penis remained stationary with not the slightest indication of edema. There was no softening of the callosities and no changes in the

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* The hormones used in this study—Alphaestradiol (Progynon-DH) and Ethinyl Estradiol (Estinyl)—were generously supplied by the Schering Corporation, Bloomfield, N. J.

contour of the perianal region. We thus failed completely to confirm in the chimpanzee the findings of Gilbert (1944) in the chacma baboon.

It is difficult to explain this failure. It seems logical to base the dosages upon the amount of the drug necessary to produce full tumescence in the castrate female. This forms the basis for the figures given in the final column of Table I. The values given for the baboon were calculated from the work of Gillman (1942) who found that 0.04 mg. estradiol benzoate in sesame oil given subcutaneously for 16 days produced maximal tumescence and for the chimpanzee from Clark (1946) who found that giving by mouth 2.0 mg. daily of alphaestradiol for 5–6 days or 0.025 mg. daily of ethinyl estradiol

TABLE I. COMPARISON OF ESTROGEN DOSAGE ADMINISTERED TO MALE CHIMPANZEE WITHOUT OBSERVABLE EFFECT ON THE SEX SKIN WITH EFFECTIVE DOSES FOR THE BABOON. DOSES ARE EXPRESSED IN MILLIGRAMS AND IN RELATION TO THE DOSE NECESSARY TO PRODUCE MAXIMAL GENITAL SWELLING IN THE FEMALE CASTRATE.

Animal	Drug	Dose per day	Length of treatment	Ratio of dose to amount (per day) necessary to produce full tumescence in female
DON	Alphaestradiol	2 mg.	49 days	1×
Castrate male	Alphaestradiol	2 mg.	29 days	1×
	Ethinyl Estradiol	0.1 mg.	8 days	4×
	Ethinyl Estradiol	0.2 mg.	8 days	8×
	Ethinyl Estradiol	4.5 mg.	12 days	180×
SHORTY	Ethinyl Estradiol	0.05 mg.	16 days	2×
Normal male	Ethinyl Estradiol	0.25 mg.	16 days	10×
	Ethinyl Estradiol	1.25 mg.	14 days	50×
	Ethinyl Estradiol	4.5 mg.	12 days	180×
BABOON*	Estradiol Benzoate	1.0 mg.	12–15 days	25×
Normal	Estradiol Benzoate	5.0 mg.	10–12 days	125×
Castrate	Estradiol Benzoate	0.1 mg.	7–15 days	2.5×

* From Gilbert (1944) and Gillman (1942).

for 6–7 days was sufficient to ensure a full swelling. The amount of estrogen given the normal male chacma baboons is seen to be 25 and 125 times the effective dose for the female castrate baboon while for the castrate baboon only 2.5 times the female dose was adequate. It will be noted that we have used both smaller and larger doses in our chimpanzees with no effect on the sex skin. It does not seem probable that the difference in results could be based upon the exact estrogens used or upon the different routes of administration since the dosages were based on the proved ability of the hormones to produce tumescence of the sex skin in the female castrates. The size and extent of the swollen sex skin of both the adult female chacma baboon and the adult female chimpanzee is truly remarkable and far surpasses that seen in other primates. Similarity in response of the sex skin of the males was thus to be expected and renders the trite explanation of species difference very unsatisfactory. Future work may afford an explanation for this difference but until this is done generalizations concerning the effects of estrogens on the sex skin seem unwarranted.

SUMMARY

In marked contrast to the baboon, changes in the sex skin of either the intact male chimpanzee or of the prepubertally castrate male chimpanzee are not produced by estrogens even when given at extremely high levels.

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EFFECTS OF PROPYLTHIOURACIL AND OF POTASSIUM THIOCYANATE ON THE UPTAKE OF IODINE BY THE THYROID GLAND OF THE RAT

Among the substances discovered to cause goiter in experimental animals propylthiouracil has been found the most potent (Astwood, Bissell and Hughes, 1945) and has been used in a considerable number of thyrotoxic patients (Astwood and VanderLaan, 1945) without producing any untoward side effects. Potassium thiocyanate has been widely used clinically in the symptomatic treatment of hypertension, and, in addition, this substance is goitrogenic both in animals and in man. Because of the clinical and physiological interest attaching to the effects of these substances many observations have been made on their influences on the discharge and uptake of iodine by the thyroid gland. As these observations have led to diverse conclusions regarding the mechanisms by which these substances produce goiters the experiments herein reported have been undertaken.

Astwood and Bissell (1944) have shown that the administration of antithyroid substances to animals results in a fall in the thyroid gland iodine content concomitant with the enlargement of the gland. In the instance of thiourylene and of aminobenzene derivatives these effects occur despite the presence of moderate amounts of iodine in the diet. Astwood (1944–45) has shown that thyroid glands depleted of iodine are capable of trapping large quantities of iodine when potassium iodide is injected in the intact animal, and McGinty (1946) has noted that increasing the amount of dietary iodine raises the iodine content of the thyroid glands of rats treated with thiouracil but this does not increase the protein-bound iodine fraction. Astwood (1943) has also shown that the goitrogenic effect of thiocyanate is markedly influenced by the iodine content of the diet, the substance being much more effective in conjunction with a low iodine diet and ineffective in the presence of added iodine.

Rawson, Tannheimer and Peacock (1944) working with radioactive iodine have found the thyroid gland of rats unable to trap iodine in the presence of

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thiouracil and increasingly avid for iodine after the administration of thiocyanate. Franklin, Lerner and Chaikoff (1944) also found that thiouracil depresses the uptake of injected iodine by the thyroid gland of the rat, although in tissue slices Franklin, Chaikoff and Lerner (1944) found that the accumulation of iodine is interfered with by thiocyanate but not by thiouracil.

The present experiments were done to study the time relationship involved in the uptake and discharge of injected iodide by the thyroid gland of the rat and to study possible differences in the effects of thiouracil and of

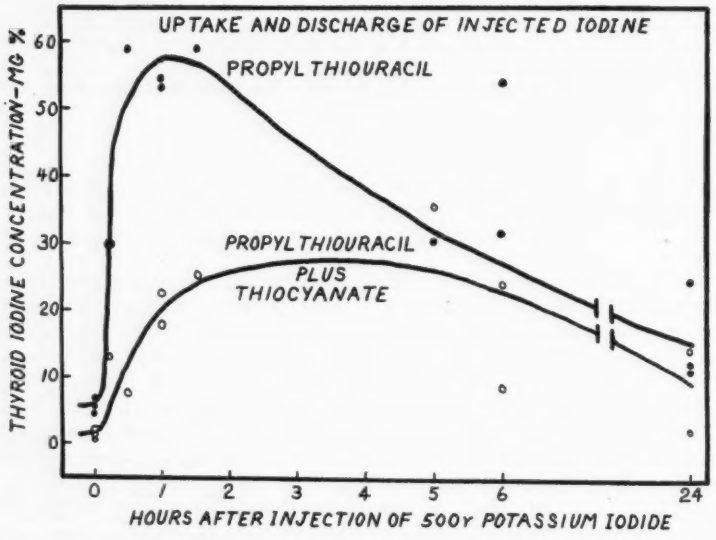

FIG. 1. The effects of propylthiouracil and of propylthiouracil plus potassium thiocyanate on the uptake and discharge of iodine after the injection of 500 ug. of potassium iodide.

thiocyanate on iodine uptake. Iodine was determined by the method of Astwood and Bissell (1944). It was considered desirable for this study to have maximal depletion of the thyroid gland iodine so that the amount of iodine trapped after injection could be determined accurately, and each iodine determination was done on the pooled thyroid glands of two rats.

Young rats were fed 0.1% propylthiouracil in the diet for 10 to 14 days. This amount of propylthiouracil is well above the maximally effective dose. 500 micrograms of potassium iodide was injected subcutaneously and the effect on the iodine content of the thyroid glands was determined for a period up to 24 hours, propylthiouracil being continued throughout. Under these conditions there was rapid uptake of iodine by the thyroid gland, reaching a maximum in about 30 minutes, followed by a discharge of the trapped iodine so that in 5 to 6 hours about one-half was lost and after 24 hours the thyroid iodine content had nearly returned to the original low level. These results indicate clearly that the thyroid gland of the rat under the influence

of propylthiouracil readily takes up injected iodine but is able to retain this iodine in the gland for only a short time. This rapid loss of iodine may be related to the fact that the accumulated iodine is not incorporated into thyroid hormone.

The above experiment was modified to study the effect of thiocyanate on the iodine-trapping mechanism. Propylthiouracil (0.1%) was again given in the diet for 10 to 14 days and in the last three days 0.3% potassium thiocyanate was given in addition, and the mixture continued to the end of the experiment. Under these conditions the injection of potassium iodide was followed by a delayed and only moderate uptake of iodine by the thyroid gland. It is clear that in the presence of thiocyanate the ability of the thyroid

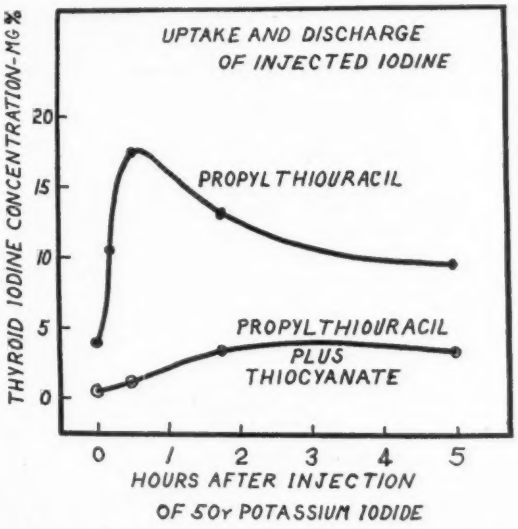

FIG. 2. The effects of propylthiouracil and of propylthiouracil plus potassium thiocyanate on the uptake and discharge of iodine after the injection of 50 ug. of potassium iodide.

gland depleted of iodine to take up injected iodine is considerably impaired. This was further confirmed in experiments where no thiocyanate was added to the diet but a single injection of potassium thiocyanate was given two hours before the injection of potassium iodide. When, instead of 500 micrograms, 50 microgram doses of potassium iodide were used the inhibiting effect of thiocyanate was even more striking. Typical experiments are shown in figures 1 and 2. It cannot be said from these experiments that thiocyanate renders the thyroid gland unable to retain iodine, as the decline may be entirely due to the influence of propylthiouracil.

It is believed that many previous reports can be harmonized on the basis of these results. Thus in many instances the conclusion that thiouracil prevents the uptake of iodine by the thyroid gland is based on determinations of iodine made several hours after this substance was injected, at which time it has already been in large part discharged. These results seem in harmony

with the observation that thiocyanate goiter is prevented by added iodine and indicate that thiocyanate interferes with the uptake of iodine on the basis of an effect which can be overcome by additional available iodine. Thiouracil, on the other hand, does not interfere with the trapping mechanism but interferes with thyroid hormone synthesis.

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